Functional Characterisation of Murine Gammaherpesvirus 68 Glycoprotein 150

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DECLARATION

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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ABSTRACT

Murine gammaherpesvirus 68 (MHV-68) is a B cell tropic pathogen of small rodents which shares genetic and pathobiological similarities with Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV). Unlike EBV and KSHV, MHV-68 replicates well *in vitro* and infects inbred mice making it a valuable and amenable model for the study of gammaherpesvirus replication and their interaction with the host. Glycoprotein 150 (gp150) is a virion membrane glycoprotein of MHV-68 that shares similarities with gp340/220 a membrane glycoprotein of EBV which facilitates EBV attachment to B cells. Antibodies against gp150 have been reported to neutralise MHV-68 infection. The aim of this study was to determine the function of gp150 and latterly to assess the potential of gp150 as a vaccine antigen to prime and protect inbred mice against MHV-68 infection. For functional studies of gp150 two main strategies were adopted; (i) the production of a recombinant virus in which the gene encoding gp150 is made dysfunctional resulting in a gp150 'knockout' (KO) virus and (ii) generation and use of purified gp150 in cell binding studies to determine if gp150 can bind to cells. Recombinant viruses were generated; virus induced plaques expressing the green fluorescent protein, used as a marker gene for identification of recombinant viruses, were observed. However, no viruses in which the required deletion of the gp150 gene had occurred were isolated. A gp150-His fusion protein (gp150-His) consisting of the extracellular domain of gp150 attached to a hexahistidine residue was successfully cloned, expressed in bacteria and purified. Similarly, a glutathione-S-transferase-His (GST-His) fusion protein was generated to be used as a control in binding studies. No significant binding of gp150-His to murine epithelial cells was detected in an enzyme linked immunosorbent assay (ELISA) or by fluorescent associated cell sorting (FACS) analysis. In contrast, significant binding of gp150-His to primary splenocytes was shown by FACS analysis. Gp150-His also bound to purified splenic B cells and both $CD19^+$ (B cells) and $CD19^-$ splenocytes. Antibodies against gp150 failed to block binding of MHV-68 to murine epithelial cells. Results indicate that gp150-His binds the heterogeneous splenic cell population as a whole i.e. not a particular subset of lymphocytes. This suggests gp150 may interact with a ubiquitous cell surface protein or perhaps a protein specific to leukocytes and could be involved in MHV-68

attachment to these cells. Gene gun nucleic acid immunisation of inbred mice with a plasmid encoding gp150 under the control of a constitutive promoter, alone or in combination with a recombinant vaccinia virus expressing gp150 (VV^{gp150}) was undertaken followed by intranasal challenge with MHV-68. Virus specific antibody appeared earlier in the group that received gp150 DNA plus VV^{gp150}. The groups that received gp150 DNA in conjunction with either VV^{gp150} or a control vaccinia virus (VV^{gpt}) appeared to have reduced levels of latently infected cells in the spleen day 15 post infection and reduced splenomegaly (a phenomenon of MHV-68 infection) in comparison with control mice. This could indicate that vaccinia virus, in a non-specific manner, boosts the specific immune response to previously administered DNA and in this case was able to limit the level of MHV-68 reaching the spleen. However, this vaccine regimen failed to significantly alter the level of infectious virus in the lung or prevent the establishment of latent virus in the spleen.

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ABBREVIATIONS

AHV	alcelaphine herpesvirus
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
Asn-X-Ser/Thr	Asparagine-X-Serine/Threonine
BBS	borate buffered saline
BCBL	body cavity-based lymphoma
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHK	baby hamster kidney
BHV	bovine herpesvirus
BL	Burkitt's lymphoma
bp	base pairs
BSA	Bovine serum albumin
^{o}C	degrees centigrade
CD	cluster of differentiation
cDNA	complementary DNA
CMV	cytomegalovirus
CMVIE	CMV immediate early
CR	complement receptors
CRP	complement regulatory proteins
CsCl	caesium chloride
CTL	cytotoxic T lymphocyte
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
Dol	dolichol

ds	double stranded
dTTP	deoxythymidine triphosphate
dUTPase	deoxyuridine triphosphatease
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBNA LP	EBNA leader protein
eBL	endemic Burkitt's lymphoma
EBV	Epstein-Barr virus
E. coli	*Eschericia coli*
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
EHV	equine herpesvirus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	Fluorescence associated cell sorter
FCS	foetal calf serum
FGARAT	*N*-formylglycinamide ribonucleotide amidotransferase
FITC	fluoresceine isothiocyanate
g	gram
gB(C/D/E/G/H/J/L/M/N)	glycoprotein B (C/D/E/G/H/J/L/M/N)
GCR	G protein coupled receptor
GDP	guanosine diphosphate
GFP	green fluorescent protein
Glc	glucose
GlcNAc	N-acetyl glucosamine
gp	glycoprotein
GST	glutathione-S-transferase
HCl	hydrochloric acid
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HHV	human herpesvirus
HIV	human immunodeficiency virus
His	histidine

HLA	human leukocyte antigen
HPX	horseradish peroxidase
HSV	herpes simplex virus
HV	herpesvirus
Hve	herpesvirus entry mediator
HVS	herpesvirus saimiri
IC	infectious centre
ICAM	intercellular adhesion molecule
IFN	interferon
IgA(E/G/M)	immunoglobulin A (E, G or M)
IL	interleukin
IM	infectious mononucleosis
in	intra-nasal
ip	intra-peritoneal
IPTG	isopropyl-β-D-thiogalactopyranoside
IR	internal repeat
IRF	interferon regulatory factor
iv	intra-venous
kbp	kilobase pair
kb	kilobase
KCl	potassium chloride
kDa	kilo Daltons
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma associated herpesvirus
l	litre
LCL	lymphoblastoid cell line
LFA	lymphocyte function-associated antigen
LMP	latent membrane protein
LPD	lymphoproliferative disease
LUR	long unique region
µg	microgram
µl	microlitre

M	molar (moles per litre)
mA	milli Amperes
MACS	magnetic associated cell sorting
Man	mannose
MCD	multicentric Castleman's disease
MCF	malignant catarrhal fever
MCP	major capsid protein
MDV	Marek's disease herpesvirus
mg	milligram
MHC	major histocompatibility complex
MHV-68	murine gammaherpesvirus 68
MIP	macrophage inflammatory protein
ml	millilitre
MOI	multiplicity of infection
MRNA	messenger RNA
MW	molecular weight
NaCl	sodium chloride
NBT	nitro blue tetrazonium
NCAM	neural cell adhesion molecule
NCS	new-born calf serum
NGS	normal goat serum
Ni^{2+}	nickel
NK	natural killer
NPC	nasopharyngeal carcinoma
NPS	normal pig serum
NRS	normal rabbit serum
OD	optical density
OHV	ovine herpesvirus
ORF	open reading frame
ori	origin of replication
PAS	protein-A-sepharose
PBS	phosphate buffered saline

PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
pfu	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
PTLD	post transplant lymphoproliferative disease
RNA	ribonucleic acid
R-PE	R-phycoerythrin
RSV	respiratory syncytial virus
RR	ribonucleotide reductase
RT	room temperature
RT-PCR	reverse transcription PCR
sBL	sporadic Burkitt's lymphoma
SCID	severely combined immunodeficient
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TAE	tris-acetate ETDA
TBE	tris-borate EDTA
TBS	tris buffered saline
TEMED	N, N, N', N'-Tetramethylethylenediamine
TK	thymidine kinase
TNE	tris normal saline ETDA
TPB	tryptose phosphate broth
TR	terminal direct repeat
TS	thymidine synthase
UDP	uridine diphosphate
UV	ultra violet
V/V	volume per volume
VZV	varicella-zoster virus
WT	wild type

W/V	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
x g	gravitational force
XLP	X-linked lymphoproliferative syndrome
ZEBRA	Z fragment Epstein-Barr reactivation antigen

1.1.1 An Introduction to Herpesviruses

Herpesviruses are large double stranded deoxyribonucleic acid (DNA) viruses widespread throughout nature, attempts to isolate them for most animals; including fish, amphibians, reptiles, birds and mammals, have been successful. To date, eight human herpesviruses have been identified. Herpes simplex virus 1 and 2 (HSV 1 and HSV2) and varicella-zoster virus (VZV) are human alphaherpesviruses. These viruses undergo acute and recurrent infections of the epidermal skin tissue resulting in the development of cold sores and genital herpes (HSV), chicken pox and shingles (VZV) (Whitley, 1996, Arvin, 1996). Complications of alphaherpesvirus infections can result in encephalitis, meningitis and keritinitis (Whitley, 1996). Human cytomegalovirus (HCMV), Human herpesvirus 6 (HHV6), Human herpesvirus 7 (HHV7) are betaherpesviruses their infection of man is largely asymptomatic infections (Britt & Alford, 1996, Pellet & Black, 1996, Frenkel & Roffman, 1996). There is on going debate as to whether HHV6 is the etiology of a number of human diseases including multiple sclerosis. Primary betaherpesvirus infection can result in mononucleosis like syndromes and mild skin rashes. Vertical transmission to the foetus can result in a variety of deformities such as blindness, hearing loss and mental retardation. Infected individuals who are immunocompromised either through receiving immunosuppressive therapies or who have acquired immunodeficiency syndrome (AIDS) can develop lethal disseminated infections (Britt & Alford, 1996, Pellet & Black, 1996). Epstein-Barr virus (EBV) and Kaposi's sarcoma virus (KSHV) also known as human herpesvirus 4 (HHV4) and human herpesvirus 8 (HHV8) respectively, are gammaherpesviruses. Gammaherpesvirus infections are largely asymptomatic. However, EBV is associated with the syndrome infectious mononucleosis and a number of malignancies such as nasopharyngeal carcinoma (NPC), endemic Burkitt's lymphoma, post transplant lymphoproliferative disease and Hodgkin's lymphoma (Rickinson & Kieff, 1996). The relatively recently discovered HHV8 has been shown to be associated with Kaposi's sarcoma and body-cavity based B cell lymphomas (Chang *et al.*, 1994, Cesarman *et al.*, 1995a, Soulier *et al.*, 1995). The prevalence of these conditions is greatly increased in immunocompromised individuals.

An important common biological property of herpesviruses is their ability to establish latent infection; the viral genome becomes episomal and a restricted set of viral genes is expressed. This property of herpesviruses enables their lifelong persistence within the host. The limited number of viral proteins expressed enables the virus to evade immune detection. The latent infection may subsequently be reactivated resulting in the production of infectious virions (Roizman, 1996).

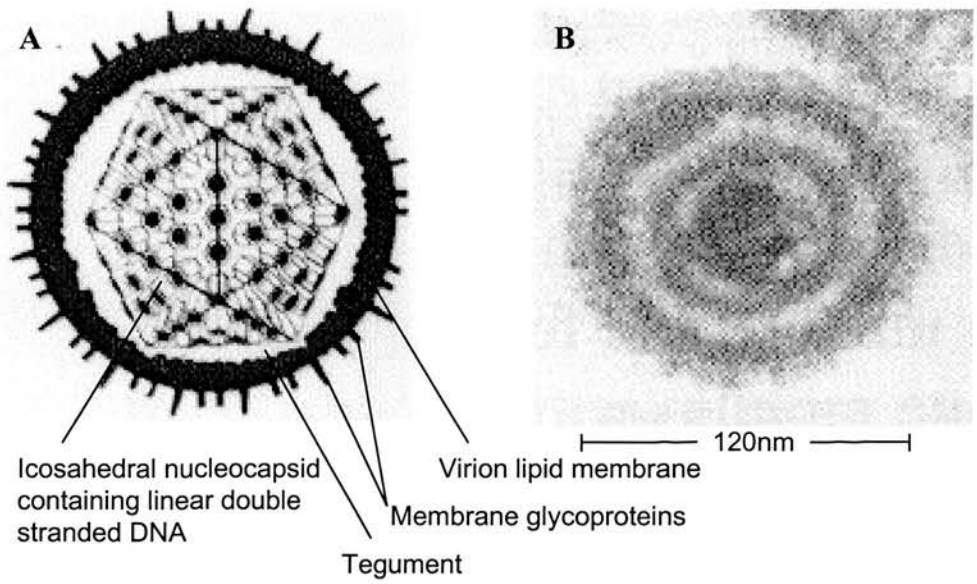
1.1.2 Herpesvirus Composition

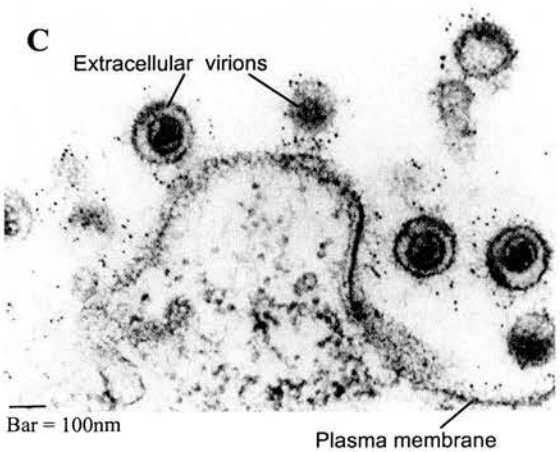
A common morphology determines herpesvirus designation. Typically, herpesvirus virions consist of a core containing linear double stranded DNA in a toroid form enclosed by an icosahedral capsid, approximately 100nm in diameter, formed from 162 capsomeres with a channel running through the centre. A 'tegument' consisting of amorphous material surrounds the capsid. A lipid envelope forms the outer covering of the virion which contains many glycoproteins that protrude, in a spike like fashion, from the virion surface (refer to figure 1). Among studied herpesviruses the DNA varies in size from approximately 125 -235 kbp and commonly encodes around 80 open reading frames (Gompels *et al.*, 1988, Roizman, 1996). The genomic organisation of the 80 plus vertebrate herpesviruses differs between types in the number and positioning of repeat sequences interspersed by unique regions (Roizman, 1996).

1.1.3 Herpesvirus Subfamilies

Despite common properties displayed by the family, herpesviruses vary widely with respect to host range, replication rate, efficiency of cell destruction, cell specificity and clinical manifestations. Based on biological properties and, more recently, genetic conservation three herpesvirus subfamilies have been defined; alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae (Roizman, 1996). Table 1.1 displays examples of herpesviruses from each subfamily.

Figure 1. Structure & appearance of herpesvirus virions





A. A schematic representation of the herpesvirus virion (Adapted from Roizman, 1996). **B.** An electron micrograph of a section through a herpes simplex virion (Adapted from Levy *et al.*, 1994). **C.** An electron micrograph of extracellular Epstein-Barr virus (EBV) virions budded from EBV-infected lymphocytes, the black dots are 5nm-diameter gold particles staining the membrane glycoprotein gp340/220 (From Gong & Kieff, 1990).

Alphaherpesvirus characteristics include a variable host range, short replication cycle, rapid spread in cell culture and their primary site of latency in sensory ganglia.

Betaherpesviruses have a restricted host range, a longer replication cycle and spread slowly in culture. A notable characteristic of betaherpesvirus infection is the enlarged phenotype of infected cells with intranuclear inclusions. Latency is established in secretory glands, lymphoreticular cells, kidneys and other tissues.

Gammaherpesviruses have a very restricted host range, limited to the family or order of the natural host. *In vitro* some gammaherpesviruses can cause lytic infection in types of epithelial and fibroblastic cells, all members are able to replicate in lymphoblastoid cells. They are either T or B cell specific and characteristically establish latency in these lymphocytes commonly within lymphoid tissues. There are two genera the *Lymphocryptovirus,* also called gamma1herpesvirus, for which EBV is the prototype, and *Rhadinovirus*, alternatively named gamma2herpesvirus, the protoypical member being herpesvirus saimiri (HVS). Other gammaherpesviruses include KSHV/HHV8, bovine herpesvirus 4 (BHV-4), ovineherpesvirus 2 (OHV-2), alcelaphine herpesvirus 1 (AHV-1) equine herpesviruses 2 and 5 (EHV2 and EHV5) and murine gammaherpesvirus 68 (MHV-68) (Roizman, 1996).

Name	Host	Size	Disease association
ALPHAHERPESVIRUS subfamily			
Herpes simplex subgroup			
Herpes Simplex 1 (HSV-1/HHV1)	Human	152	Cold sores, keratitis, ocular disease
Herpes Simplex 2 (HSV-2/HHV2)	Human	152	Genital herpes
Bovineherpes virus 2 (BHV-2)	Cow	133	Rhinotracheitis
Marek's Disease Virus (MDV)	Chicken	180	T cell lymphomas in chickens
Varicellovirus subgroup			
Varicella zoster (VZV/ HHV3)	Human	125	Chicken pox, shingles
Pseudorabies virus	Pig	140	Aujeszky's disease
Equine herpesvirus 1	Horse		Respiratory disease, abortion
Salmonid herpesvirus 1	Fish		Epithelial tumours
BETAHERPEVIRUS subfamily			
β1;Cytomegalovirus subgroup			
Human cytomegalovirus (HCMV/ HHV5)	Human	229	Foetal deformatities Lymphoproliferation in immunocompromised
Herpesvirus aotus type 1	Primates	220	
Murine cytomegalovirus (MCMV) Murid herpesvirus 1	Mouse	235	
β2;Roseolovirus subgroup			
Human herpesvirus 6 (HHV6)	Human	162	
Human herpesvirus 7 (HHV7)	Human		
GAMMAHERPESVIRUS subfamily			
γ1; Lymphocryptovirus subgroup			
Epstein-Barr virus (EBV)/ (HHV-4)	Human	172	Infectious mononucleosis Burkitt's lymphoma Nasopharyngeal carcinoma Hodgkin's lymphoma
Herpesvirus pan	Chimpanzee	170	
Herpesvirus papio	Baboon	170	
γ2; Rhadinovirus subgroup			
Herpesvirus saimiri	Squirrel monkey	155	Fatal lymphoproliferations in cotton tail rabbit & other New World monkeys e.g. marmosets
Kaposi's sarcoma associated herpesvirus (KSHV)/ Human herpesvirus 8 (HHV-8)	Human	140	Kaposi's sarcoma Body cavity based lymphoma Multicentric Castlemans disease
Herpesvirus ateles	Spider monkey	135	
Equine herpesvirus 2	Horse	184	Respiratory disease
Equine herpesvirus 5	Horse	150	Respiratory disease
Herpesvirus sylvilagus	Cotton tail rabbit		Lymphomas & lymphoproliferations
Woodchuck herpesvirus	Woodchuck	160	
Murine gammaherpesvirus 68/ Murid herpesvirus 4	Murid rodents	118	Lymphomas
Bovine herpesvirus 4	Cow	145	Respiratory disease
Wildebeest herpesvirus/ Alcelaphine herpesvirus 1	Wildebeest	160	Malignant Catarrhal fever in cattle
Ovine herpesvirus 2	Sheep		Malignant catarrhal fever in cattle

Table 1.1. Examples of members of each herpesvirus family. **Size**; approximate size of genome in kbp. Note, this represents only a limited number of herpesviruses and is in no way proportional to the number of members known for each subfamily, more gammaherpesviruses are displayed due to greater relevance to this document. HHV; Human herpesvirus. (Adapted from Roizman, 1996).

1.1.4 Genomic Content – Conserved Gene Blocks

Within the unique DNA of herpesvirus genomes the reading frames are closely arranged and they frequently overlap. There are both leftward and rightward orientated genes and genes containing splice sites. A set of conserved genes is carried by most herpesviruses and these encode products required for productive viral replication. These range from enzymes required for nucleic acid metabolism, (such as thymidine kinase, alkaline exonuclease, dUTPase and the large subunit of ribonucleotide reductase), enzymes for DNA synthesis (for example DNA polymerase and helicase-primase), transcriptional activators, glycoproteins and structural capsid proteins. Initially three conserved gene blocks were defined (Davison & Taylor, 1987), this has now been expanded to seven regions of conserved genes (Chee *et al.*, 1990). Table 1.2 displays the blocks of conserved and semiconserved genes of the herpesviruses. The blocks of conserved genes are interspersed with blocks of genes specific to a viral family or the specific virus.

1.1.5 Unique Genes

Out with the conserved herpesvirus genes there are a variety of types of unique genes encoded by individual herpesviruses which have evolved within viruses for adaptation to particular environments or confer a particular advantage on the virus. Some unique genes are specific to one particular virus whereas others appear to be pertained by a few different herpesviruses. There are many examples of viral genes which have cellular homologues these encode proteins with roles in modulation of cell cycle and signal tranduction e.g. cyclin D, bcl-2 and G-protein coupled receptor ($IL\text{-}8^R$); cytokines, chemokines and modulators of host immune response e.g. IL-10, IL-17 and complement control proteins. Although there is a set of highly conserved herpesvirus glycoproteins specific viruses also encode unique glycoproteins of which gp150 of MHV-68 is considered to be one (discussed further in section 1.7.7). Although all studied herpesviruses have genes associated with latency between different viruses these genes are very diverse and lack conservation (Virgin *et al.*, 1997, Albrecht *et al.*, 1992, Russo *et al.*, 1996, Baer *et al.*, 1984).

Table 1.2 Conserved gene blocks of herpesviruses

Gene Block	Gene Function	α-Herpesviruses		β-Herpesviruses		γ-Herpesviruses	
		HSV-1	VZV	HCMV	HHV7	HVS	EBV
1	DNA replication protein	UL42	ORF16	UL44	UL27	ORF59	BMRF1
1	RR (small)	UL40	ORF18			ORF60	BARF1
1	RR (large)	UL39	ORF19	UL45	UL28	ORF61	BORF2
1	Capsid assembly/DNA maturation protein	UL38	ORF20	UL46	UL29	ORF62	BORF1
1	Myosin, Tegument protein	UL37	ORF21	UL47	UL30	ORF63	BOLF1
1	Tegument protein	UL36	ORF22	UL48	UL31	ORF64	BPLF1
1	Capsid protein	UL35	ORF23	UL49	UL33	ORF66	BFRF2
1	Tegument protein	UL34	ORF24	UL50	UL34	ORF67	BFRF1
1		UL33	ORF25	UL51	UL35		
1	Glycoprotein	UL32	ORF26	UL52	UL36	ORF68	BFLF1
1		UL31	ORF27	UL53	UL37	ORF69	BFLF2
2	DNA polymerase	UL30	ORF28	UL54	UL38	ORF9	BALF5
2	Glycoprotein B	UL27	ORF31	UL55	UL39	ORF8	BALF4
2	Transport protein	UL28	ORF30	UL56	UL40	ORF7	BALF3
2	ssDNA binding protein	UL29	ORF29	UL57	UL41	ORF6	BALF2
3	IE transactivator	UL54	ORF4	UL69	UL42	ORF57	BMLF1
3	Helicase/primase	UL52	ORF6	UL70	UL43	ORF56	BSLF1
3		UL51	ORF7	UL71	UL44	ORF55	BSRF1
3	dUTPase	UL50	ORF8	UL72	UL45	ORF54	BLLF2
3	Membrane protein (gN)	UL49a	ORF9a	UL73	UL46	ORF53	BLRF1
4	Glycoprotein H	UL22	ORF37	UL75	UL48	ORF22	BXLF2
4	Thymidine kinase	UL23	ORF36			ORF21	BXLF1
4	Fusion protein	UL24	ORF35	UL76	UL49	ORF20	BXRF1
4	Tegument protein	UL25	ORF34	UL77	UL50	ORF19	BVRF1
4				UL79	UL52	ORF18	BVRF1.5
4	Capsid/assembly protein	UL26	ORF33	UL80	UL53	ORF17	BVRF2
5	Capsid protein	UL18	ORF41	UL85	UL56	ORF26	BDLF1
5	Major capsid protein	UL19	ORF40	UL86	UL57	ORF25	BcLF1
5				UL87	UL58	ORF24	BcRF1
6	DNA packaging protein	UL15	ORF42	UL89^{c2}	UL60	ORF29b	BDRF1
6				UL92	UL63	ORF31	BDLF
6		UL17	ORF43	UL93	UL64	ORF32	BGLF1
6		UL16	ORF44	UL94	UL65	ORF33	BGLF2
6	DNA packaging protein	UL15	ORF45	UL89^{c1}	UL66	ORF29a	BGRF1
6		UL14	ORF46	UL95	UL67	ORF34	BGLF3
6				UL96	UL68	ORF35	BGLF3.5
6	Kinase	UL13	ORF47	UL97	UL69	ORF36	BGLF4
6	Alkaline exonuclease	UL12	ORF48	UL98	UL70	ORF37	BGLF5
6	Glycoprotein M	UL10	ORF50	UL100	UL72	ORF39	BBRF3
6	Helicase/primase	UL8	ORF52	UL102	UL74	ORF41	BBLF3
6		UL7	ORF53	UL103	UL75	ORF42	BBRF2
6	Capsid protein	UL6	ORF54	UL104	UL76	ORF43	BBRF1
6	Helicase/primase	UL5	ORF55	UL105	UL77	ORF44	BBLF4
7	Uracil DNA glucosidase	UL2	ORF59	UL114	UL81	ORF46	BKRF3
7	Glycoprotein L	UL1	ORF60	UL115	UL82	ORF47	BKRF2

Conserved gene blocks of alpha, beta and gammaherpesviruses. Grey shading indicates partially conserved genes. Note that the order of the gene blocks follows the betaherpesvirus arrangement. RR – ribonucleotide reductase, IE – immediate early. HSV-1 – Herpes simplex virus 1, VZV – varicella zoster virus, HCMV – human cytomegalovirus, HHV7 – human herpesvirus 7, HVS – herpesvirus saimiri, EBV – Epstein-Barr virus.

1.1.6 Features of Herpesvirus Lytic Replication

Much of the information on herpesvirus lytic replication has been established from the study of herpes simplex virus. The course of herpesvirus lytic replication follows a general pattern of events; initial attachment to target cell, entry, uncoating of virus capsid, trafficking of viral DNA to the nucleus, viral gene expression, viral DNA replication, assembly of capsid and packaging of DNA, envelopment of the capsid in a membrane studded with viral proteins, egress and budding from cell (Roizman & Sears, 1996).

The viral envelope enables entry of the virus into cells, glycoproteins within the envelope membrane facilitate the attachment to the target cell and the post attachment entry process. The herpesvirus envelope either fuses, in a pH-independent manner, directly with the cellular membrane or with the vesicular membrane following capping of the virion into the cell. The capsid acts as a vector for the viral DNA trafficking it to the cell nucleus. For all herpesviruses DNA synthesis and capsid construction occurs in the nucleus of infected cells. When the viral genome is released by the capsid into the nucleus it immediately circularises. It is then replicated by a rolling circle mechanism which yields head to tail concatemers of unit length (Jacob & Roizman, 1977, Jacob *et al.*, 1979). Sequential expression of immediate early, early and late genes occurs (described in section 1.1.7). Several proteins required for DNA synthesis e.g. DNA polymerase, alkaline exonuclease, dUTPase and ribonucleotide reductase large are virally encoded and their genes are conserved across the herpesviruses (Davison & Taylor, 1987). Herpesvirus genomes contain origins of replication, HSV has three, these are sites from which DNA replication is initiated and they are essential for the herpesvirus genome to be amplified. Unit lengths of DNA are cleaved from the concatemers and packaged according to sites coded for by consensus sequences upstream of the cleavage site. Homologous sequences exist in HSV 1 and 2, EBV, HVS, BHV1, HCMV and VZV (Davison,1984, Deiss *et al*, 1986, Matsuo *et al*, 1984 and Spaete & Mocarski, 1985).

Late viral gene expression results in the production of viral glycoproteins which localise to the nuclear membrane. Following packaging of the viral genome into the capsid the capsid undergoes a conformational change and buds from the nucleus (Deiss *et al.*, 1986). The exact process by which herpesviruses gain their final envelope membrane is not entirely clear. The body of evidence suggests that in the case of HSV, immature glycoproteins embed into the nuclear membrane, and the mature capsids bud through the membrane acquiring both envelope and glycoproteins (Vlazny *et al.*, 1982). The immature virus particles, then pass through the Golgi apparatus, on route to the plasma membrane, where viral glycoproteins become processed to maturity (Desai *et al*, 1988, Kousoulas *et al*, 1983 a & b). The mature viruses travel to the plasma membrane, via a Golgi derived vesicle and are released into the extra-cellular space by a process of reverse phagocytosis (Johnson & Spear, 1982). Alternatively, the virus may acquire its final envelope at the plasma membrane where glycoproteins have been trafficked to. There is data suggesting that HSV-1 acquires it's final membrane from a post-ER compartment i.e. the Golgi and implies that the virus undergoes de-envelopment and re-envelopment steps during virus egress (Whiteley *et al.*, 1999). Figure 1.2. depicts the lytic replication cycle of EBV, in this case the capsid is thought to acquire it's envelope by budding at the plasma membrane or following envelopment by post-Golgi vesicles (Gong & Kieff, 1990). Electron micrographs have shown HHV-8 acquiring a viral glycoprotein studded envelope as it buds from the plasma membrane (Li *et al.*, 1999).

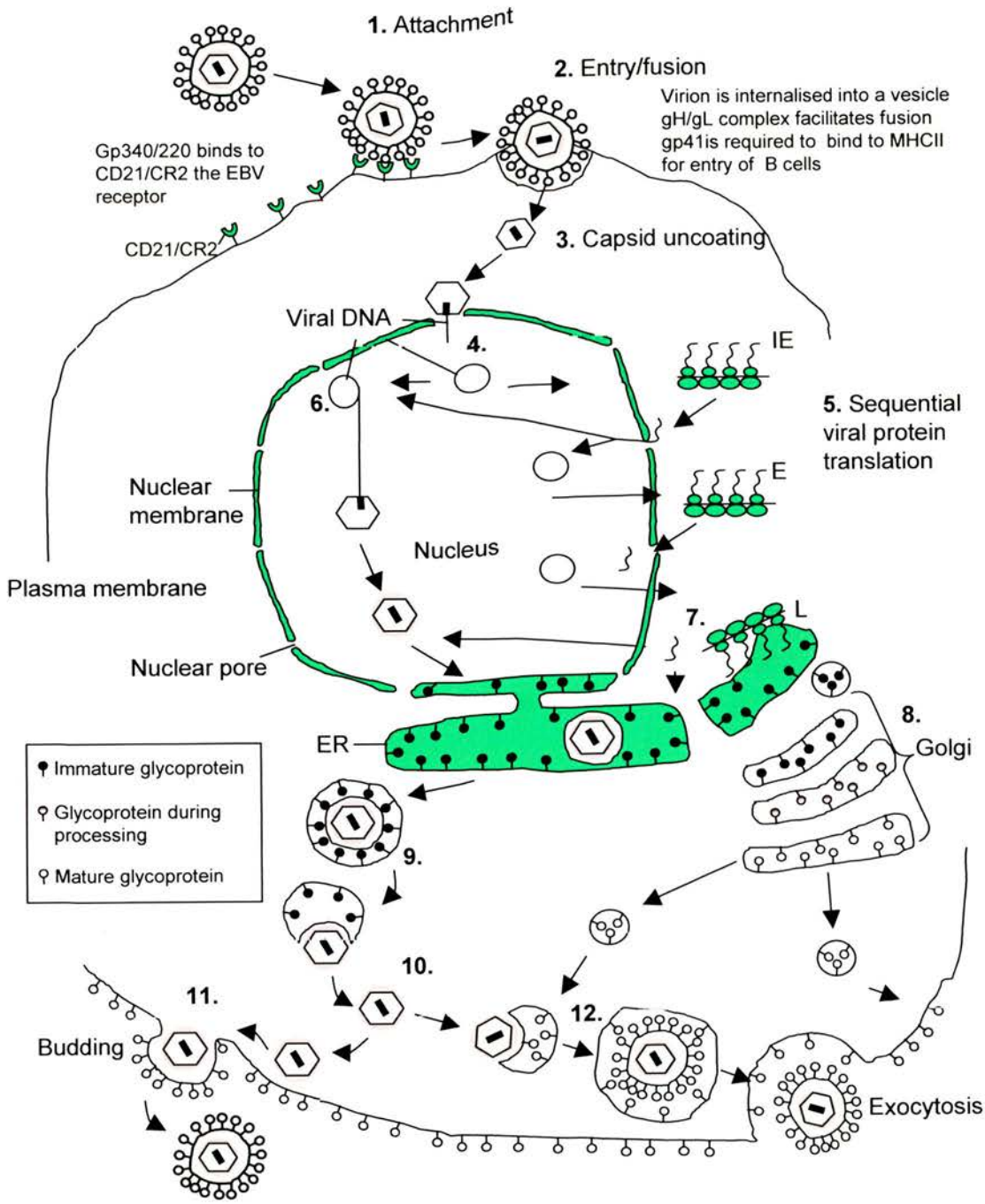


Figure 1.2. Schematic representation of the process of lytic replication of EBV. Description on following page.

Figure 1.2. Schematic representation of the process of lytic replication of EBV. **1.** The virus attaches to the target cell by interaction of a virion membrane glycoprotein with a cell surface receptor, in the case of EBV to the complement receptor CR2/CD21. **2.** Following attachment the virion is internalised into a vesicle derived from the plasma membrane, the virion membrane fuses with the vesicle membrane in a pH independent manner, membrane glycoproteins are required for this event, fusion effects the release of the viral capsid into the cytoplasm. **3.** The uncoated capsid traffics to the nuclear membrane and releases the linear double stranded viral DNA into the nucleus. **4.** The linear viral genome immediately circularises. **5.** Sequential viral protein expression ensues, initially immediate early proteins (IE) are generated these include transcriptional activators that act to initiate synthesis of the early viral proteins (E) which include enzymes involved in DNA synthesis (e.g. DNA polymerase) and nucleotide metabolism (e.g. thymidine kinase). Subsequently, late proteins (L) are expressed these include structural capsid proteins and viral glycoproteins. **6.** The viral genome is replicated via a rolling circle mechanism. Lengths of genome are cleaved and directed into newly formed nucleocapsids. Complete capsids with packaged DNA enter the endoplasmic reticulum (ER). **7.** Newly synthesized proteins enter the ER and receive modifications such as N-linked glycosylation. **8.** The majority of glycoproteins travel through the Golgi receiving further processing to newly acquired N-linked glycosylation and addition of O-linked glycosylation on their way to the cell membrane. **9.** Vesicles release nucleocapsids into the cytoplasm **10.** It is proposed that the nucleocapsid acquires a definitive envelope either by **11.** budding through the plasma membrane containing the Golgi-processed viral glycoproteins or **12.** by budding into post -Golgi derived vesicles containing viral glycoproteins followed by exocytosis of the enveloped virion. Adapted from Roizman & Sear, 1996 and Gong & Kieff, 1990.

1.1.7 Herpesvirus Gene Expression & Transcription Pattern

During a productive infection, herpesvirus gene expression is tightly regulated and occurs in a sequential fashion. The primary viral genes expressed following infection and arrival of the viral DNA in the nucleus are termed immediate early genes that are transcribed prior to viral protein synthesis. These include transcription activators that turn on other viral genes. The next set of genes expressed are termed early genes which are transcribed in the absence of viral DNA synthesis and are required to facilitate replication of the viral genome. These genes encode proteins such as additional transcriptional activators and those genes involved in nucleic acid metabolism, genome replication and capsid assembly e.g. helicase-primase, TK, DNA polymerase, dUTPase etc. Subsequently the late genes are expressed following transcriptional activation via the early expressed transcription activators e.g. the R transactivator of EBV (Ragoczy & Miller, 1999). The late genes encode structural proteins that are expressed only after viral DNA synthesis e.g. tegument proteins and glycoproteins required for the assembly of virions (Roizman & Sears, 1996).

During latent infection viral gene expression is very restricted and is initiated from promoters separate from those used during productive viral infection. When classifying genes according to their pattern of expression the herpesvirus groupings consist of latent and lytic, lytic genes being subdivided into immediate early, early and late. Herpesvirus genes are transcribed predominantly by host cell RNA polymerase II, RNA polymerase III transcribes a small proportion of genes which commonly encode non-translated RNAs.

1.1.8 Gammaherpesviruses

Of the gammaherpesviruses, most interest has been directed at those causing disease in humans (EBV & HHV8) and domestic cattle (OHV 2 and AHV 1). OHV2 and AHV1 have a causal association with malignant catarrhal fever (MCF), a fatal lymphoproliferation of domestic ruminants and are therefore economically important (Bridgen *et al.*, 1991 & 1989, Plowright *et al.*, 1972). In addition, herpesvirus saimiri has received significant research interest due to its ability to transform T lymphocytes *in vitro* (Mittrucker *et al.*, 1993) and its oncogenic effects in animals

(New World primates e.g. marmosets and the cotton tail rabbit) other than the squirrel monkey, its natural host (Medveczky *et al.*, 1989, Wright *et al.*, 1977). Gammaherpesviruses share the ability to establish latent infection in lymphocytes. Most gammaherpesviruses are B cell tropic e.g. EBV and MHV-68 however there are exceptions such as HVS which is T cell tropic.

1.1.9 Epstein-Barr Virus

EBV, the prototype gamma1herpesvirus originally isolated from an endemic Burkitt's lymphoma tumour biopsy (Epstein *et al.*, 1964) was the first herpesvirus to be fully cloned and sequenced (Dambaugh *et al*, 1980, Baer *et al*, 1984, Hatfull *et al*, 1988 & Parker *et al*, 1990). The EBV genome consists of approximately 172kb linear double stranded DNA with a GC content of 60%. At each end of the genome there are multiple direct terminal repeats of about 0.5kb forming large terminal repeats (LTRs) that flank the so called unique region of the genome. Two types of EBV – type 1 and type 2 have been defined, also known as type A and B respectively. EBV-1 is more prevalent in western societies whereas both types are isolated with an equal frequency in African countries (Zimber *et al.*, 1986). The genome of type 1 and type 2 are almost identical except for the genes which encode the latency associated nuclear proteins. Type 2 is less transforming than type 1. The difference is based on variations in two latency associated genes i.e. EBV nuclear antigens (EBNAs) 2 and 3 (Rickinson *et al.*, 1987, Rowe *et al.*, 1989).

The EBV genome encodes genes for over 90 proteins these include the full contingent of conserved and partially conserved herpesvirus genes (refer to table 1.2). There are 18 gammaherpesvirus specific genes accompanied by gamma1herpesvirus specific and EBV unique genes. The ORFs are named by their location and coding orientation within a *Bam*HI restriction digest fragment of the EBV genome (e.g. BZLF1 meaning *Bam*HI Z fragment first leftward ORF) rather than being numbered by order as other gammaherpesvirus ORFs have been labelled.

The gammaherpesvirus specific genes include a bcl-2 homologue encoded by BHRF1 which is functionally active as an early expressed gene and the EBV R

transactivator (encoded by BRLF1) (Henderson *et al*, 1993 & Tarodi *et al*, 1994). EBV encodes a second functional bcl-2 (BALF4), which inhibits apoptosis and associates with Bax and Bak as does the product of BHRF1 (Marshall *et al.*, 1999). The EBV R transactivator is a lytic gene responsible for regulating the expression of other lytically expressed viral proteins (Ragoczy & Miller, 1999).

ZEBRA (Z fragment EBV reactivation antigen) encoded by BZLF1 is also a gammaherpesvirus specific gene, a homologue has been identified in KSHV. ZEBRA is associated with reactivation from latency acting as a key immediate early transactivator for lytic virus replication and is thought to be closely related to the cellular fos/jun family of proteins (Packham *et al*, 1990). As well as inducing the reactivation of the latent EBV genome, ZEBRA also causes the induction of cellular genes such as tumour growh factor (TGF-β), a powerful immunosuppressive cytokine (Cayrol & Flemington, 1995).

The latency associated proteins, described later in section 1.2.4, are encoded by EBV unique genes, no homologues have been described for any other herpesviruses. EBV encodes an interleukin 10 (IL-10) homologue expressed during lytic EBV replication (Stewart & Rooney, 1992 and Stewart *et al.*, 1994b) which has been shown to enhance the efficiency by which EBV can infect/transform B cells *in vitro* (Stuart *et al.*, 1995). The EBV IL-10 is a lymphokine able to dampen local cellular immune responses as an antagonist to natural killer (NK) cell and T cell mediated responses (Moore *et al.*, 1990). IL-10 also promotes B cell proliferation and so may act as part of the virus induced B cell activation (Rousset *et al.*, 1992).

Unlike many of the gamma2herpesviruses, EBV does not code for a G protein coupled receptor (GCR), cyclin D or a complement regulatory protein (CRP) homologue. However, EBV does specifically up regulate two cellular GCR proteins (Birkenbach *et al*, 1993), cyclin D2 (Sinclair *et al*, 1994) and complement receptors CD23 and CR 2 (also termed CD21) (Wang *et al.*, 1990, Calender *et al.*, 1987).

With greatest relevance to this document EBV encodes a unique virion membrane glycoprotein called gp340/220 (also known as gp350/220). Gp340/220 is encoded by the *Bam*HI L fragment which is located in the centre of the genome. Gp340/220 is a heavily glycosylated protein abundant on the surface of the EBV virion envelope and facilitates attachment of EBV to cells by binding to CD21 (also known as CR2) the complement receptor predominantly expressed on B cells (Nemerow *et al.*, 1987, 1989, 1990a & 1990b). MHV-68 is the only herpesvirus to encode a gene that exhibits any significant sequence homology to gp340/220 i.e. gp150 of MHV-68 displays some limited sequence similarity (Stewart *et al.*, 1996). Gp340/220 has been shown to be identical between the two types of EBV (Lees *et al.*, 1993). Further discussion of gp340/220 is present in section 1.7.3.

1.2.1 EBV Infection of Immunocompetent Hosts

In the majority of people EBV infection is subclinical, primary infection occurring during the first three years of life. The virus is thought to be orally transmitted and approximately 90% of the human population are asymptomatic carriers. EBV specific cytotoxic T cells generated during the primary infection function to keep the life long EBV infection in check as the virus persists in a few circulating B lymphocytes (Gavioli *et al.*, 1993, Murray *et al.*, 1992, Apolloni *et al.*, 1992). Complications arise when primary EBV infection occurs in early adulthood, approximately 50% of cases result in infectious mononucleosis syndrome characterised by acute enlargement of lymphoid tissue, particularly in the cervical region. As the virus replicates in the oropharynx there is a strong antibody response to the lytic antigens of EBV, however it is not clear how important this humoral immune response is in the control of the infection. The strong cell mediated immune response that occurs is thought to be the principal means of acute infection control predominantly effected by CD8 positive T cells (Reinherz *et al.*, 1980, Tomkinson *et al.*, 1987, Lynne *et al.*, 1998).

Following resolution of primary infection all infected individuals continue to shed the virus into the saliva intermittently for life (Yao *et al.*, 1985, Sixbey *et al.*, 1984). Bone marrow and B lymphocytes in the circulating blood and lymphoid tissues are

major reservoirs for EBV (Decker *et al.*, 1996, Khan *et al.*, 1996,). These resting B cells are latently infected with EBV i.e. EBV gene expression is extremely restricted, there is no productive replication to form infectious virus rather the EBV genome is maintained within the cell in a manner designed to limit immunogenicity and therefore identification by host immune surveillance. In asymptomatic carriers there is approximately one EBV positive B cell per 10^6 B cells (Lewin *et al.*, 1987, Yao *et al.*, 1991, Khan *et al.*, 1996, Miyashita *et al.*, 1995). The sporadic reactivation of EBV and proliferation of EBV infected cells that occurs is limited by the continuous presence and immune surveillance by viral specific cytotoxic T cells (Moss *et al.*, 1979, Rickinson *et al.*, 1979, Rickinson *et al.*, 1980). The importance of this immune control is well demonstrated when transplant patients under T cell immunosuppression frequently succumb to EBV infected B cell proliferation resulting in malignant lymphomas (Hanto *et al.*, 1983).

1.2.2 EBV Tropism

EBV is B cell tropic and is known to attach via the interaction of gp340/220 with the complement receptor CR2/CD21 predominantly expressed on B cells (Nemerrow *et al.*, 1987, 1990a & 1990b, Fingeroth *et al.*, 1984). CD21 is a 145 kDa membrane glycoprotein containing 8-11 N-linked oligosaccharides which binds the C3d component of complement (Tedder *et al.*, 1984, Weis *et al.*, 1984). B lymphocyte infectability correlates with CD21 expression (Jondal *et al.*, 1976). CD21 is thought to be involved in B cell activation and may be a mediator of transmembrane signal transduction (Changelian & Fearon, 1986). Binding of EBV to CD21 on resting B cells is thought to result in B cell activation and subsequent proliferation (Sinclair *et al.*, 1994).

EBV also infects epithelial cells (Sixbey *et al.*, 1984 & 1987, Greenspan *et al.*, 1985). There is uncertainty as to what receptor the virus uses to enter epithelial cells, the CD21 epitope may be expressed very weakly on normal epithelial cells (Young *et al.*, 1986) and CD21 has been detected on some human epithelial tumour cell lines *in vitro* (Birkenbach., *et al* 1992, Billaud *et al.*, 1989, Fingeroth *et al.*, 1999) alternatively EBV perhaps uses an alternative unidentified receptor (Kim *et al.*,

1998). EBV has been detected in gastric carcinomas not expressing CD21 (Yoshiyama *et al.*, 1997). The virus may also enter epithelial cells as the result of EBV infected B cells fusing with epithelial cells (Bayliss & Wolf, 1980), direct cell to cell spread (Imai *et al.*, 1998) or as a complex with IgA (Sixbey & Yao, 1992). EBV infection of T cells is uncommon however it does occur (Kikuta *et al.*, 1988, Anagnostopoulos *et al.*, 1992), T cells have been shown to express CD21 (Fischer *et al.*, 1991). An alternative EBV receptor to CD21 present on a T cell line has also been identified (Hedrick *et al.*, 1992 & 1994).

There has been some controversy as to whether the virus shed into the saliva of infected individuals is derived from squamous epithelial cells or intraepithelial B cells (Allday & Crawford, 1988, Niedobitek & Young, 1994, Karajannis *et al.*, 1997). In the more recent studies of EBV infected cell types, detection of EBV infected epithelial cells in the tonsils or exfoliated in saliva of acutely infected individuals was not possible whereas EBV infected B cells were commonly identified in the same locations (Anagnostopoulos *et al.*, 1995, Tao *et al.*, 1995, Karajannis *et al.*, 1997). It is clear however, that *in vivo* EBV can and does infect epithelial cells. Debate surrounds whether epithelial cells are important for the life cycle of the virus.

The study of EBV has been frustrated by the fact that EBV replicates inefficiently in epithelial cells *in vitro*. By manipulating epithelial cells to express the B cell receptor, CD21, on their surface more cells are infected but the infection continues to be poor (Li *et al.*, 1992, Chodosh *et al.*, 2000). This shows that post entry requirements contribute towards viral cell tropism i.e. despite the presence of the receptor, post attachment and entry the virus needs other cell specific factors, possibly transcription factors, present in order to replicate efficiently.

1.2.3 EBV-Associated Diseases

Despite EBV infection being asymptomatic in the majority of individuals the association of EBV with a number of human malignancies has fuelled the interest in research into EBV. EBV was first isolated from and is closely associated to endemic Burkitt's lymphoma (eBL) a B cell tumour localised to the jaw principally found in equatorial Africa and Papua New Guinea (Burkitt 1970). In these areas eBL occurs at approximately 10 cases per 100,000 individuals a year, accounting for half of all childhood lymphomas. Predominantly affecting children, 98% eBL are EBV positive (zur Hausen *et al.*, 1970) and EBV is considered a necessary feature with other cofactors in the chain of events to effect malignancy. An important cofactor of eBL is hyperendemic falciparum malaria infection thought to promote the chromosomal rearrangements found in eBL B cells, which result in deregulating the expression of c-myc, an oncogene. Chromosomal translocation results in the oncogene c-myc moving from chromosome (chr) 8 to either chr14, 2 or 22 where it is positioned under the control of an immunoglobulin promoter (Leder *et al.*, 1983, Klein & Klein, 1985, Lenoir & Bornkamm, 1987).

It should be noted that besides eBL there are two other forms of this lymphoma – sporadic and AIDS-associated BL which both display pathological and cytological features of endemic BL but are frequently EBV negative (Ziegler *et al.*, 1976, Magrath, 1990, Hamilton-Dutoit *et al*, 1991b). A number of EBV positive and EBV negative B cell lines have been derived from BL tumors which are used in the study of EBV (Epstein *et al.*, 1966, Pizzo *et al.*, 1978)

The presence of EBV DNA and protein expression in nasopharyngeal carcinoma (NPC) is well documented (Niedobitek *et al.*, 1991, Wolf *et al.*, 1973, Desgranges *et al.*, 1982). NPC consists of proliferation of the squamous epithelial cells in the postnasal space. The virus DNA is associated with the malignant epithelial cells and not the infiltrating lymphocytes characteristic of the EBV associated tumours (Wolf *et al.*, 1973). Although tumour incidence is infrequent across the world in general, there are areas of significantly high incidence, particularly in south east Asia. NPC is the most common tumour in Southern Chinese males and the second most

common tumour of Southern Chinese females (Simons & Shanmugaratnam, 1982). World wide there are approximately 80,000 cases annually (Parkin *et al.*,1984) resulting in death within 5 years for a high percentage of cases. It is thought that cofactors are required for the development of NPC because the time between infection and onset of the tumour is approximately 50 years. Existence of genetic predisposition to NPC is suggested by an association of the risk of development of NPC with certain HLA haplotypes (Simons *et al.*, 1975) and the high predisposition to NPC retained by first generation offspring of Southern Chinese immigrants in the USA (Ho, 1975). Environmental cofactors have been implicated in tumour development such as tumour promoting chemicals e.g. phorbol esters and nitrosamines detected in Chinese salted fish and other food products in areas of high NPC incidence (Poirier *et al.*, 1987).

EBV is also the cause of lymphoproliferative disease to which immunosuppressed individuals are susceptible. This is particularly a problem for transplant recipients due to the use of immunosuppressive drugs inhibiting T cell activity, post transplant lymphoproliferative disease (PTLD) develops as a result of uncontrolled proliferation of EBV positive B cells (Crawford *et al.*, 1980, Nalesnik *et al.*, 1988, Thomas *et al.*,1991). In lung transplant patients the incidence of PTLD has been reported to be between 6.4 – 20% (Levine *et al.*, 1999). AIDS patients frequently develop large-cell lymphomas and Burkitt's lymphoma with which EBV is associated (Beral *et al.*, 1991b, Neri *et al.*, 1991, MacMahon *et al.*, 1991).

Many AIDS patients and HIV carriers develop oral hairy leukoplakia, a wart like lesion on the lateral borders of the tongue with an epithelial focus of EBV replication. Viral causation appears clear as treatment with the antiviral drug acyclovir, which inhibits EBV replication, results in regression of the lesions, lesions return following withdrawal of the drug (Greenspan *et al.*, 1985, Sandvej *et al.*, 1992).

Individuals with a rare genetically determined, X-linked immunodeficiency are predisposed to EBV associated disease, and commonly develop X-linked lymphoproliferative syndrome also known as Duncan syndrome (Purtilo *et al.*, 1975, Harada *et al.*, 1982, , Sayos *et al.*, 1998). After acute EBV infection, the majority of males with Duncan syndrome develop fatal infectious mononucleosis, those that survive frequently develop lymphoma and/or hypogammaglobulinemia (Tatsumi & Purtilo,1986, Purtilo *et al.*, 1989).

Detection of EBV presence in up to 50% of cases has suggested EBV may play a role in Hodgkin's disease (HD) although the evidence as yet is not sufficient to implicate EBV in the aetiology of HD (Weiss *et al.*, 1989, Herbst *et al.*, 1991, Rickinson and Kieff, 1996). Hodgkin's disease is characteried by the presence of malignant multinucleate Reed-Sternberg and mononucleate Hodgkin cells within the affected lymph node. The HD tumour is thought to be of immature lymphoid origin. Within five years of infectious mononucleosis there is approximately a 5 fold increase in the likelihood of developing HD (Mueller *et al.*, 1989).

Varying frequencies of the presence of EBV DNA have been detected in different types of T cell lymphoma such as angioimmunoblastic lymphadenopathy (Ott *et al.*, 1992) nasal lymphoma (Harabuchi *et al.*, 1990) and peripheral T cell lymphoma (Jones *et al.*, 1988) however, the role of the virus in disease development remains to be elucidated. EBV has also been detected in gastric carcinomas, investigations are currently underway to elucidate if EBV has a causal role in these malignancies (Takano *et al.*, 1999, Shibata & Weiss, 1992).

Although the majority of the world population experience an asymptomatic EBV infection the occurrence of the diseases described above, particularly the syndromes closely associated with EBV such as eBL, and NPC call for a strategy to prevent EBV induced disease. As yet there is no licensed EBV vaccine, however work in this direction has been pursued for over twenty years. Sections 1.8.6-1.9.1 describe the strategies that have been approached in the generation of vaccines to prevent EBV infection.

1.2.4 EBV Latency

The ability to form a latent infection in lymphocytes is a feature common to all gammaherpesviruses and pivotal for their life long persistence within the host. To understand how this is achieved investigation into the genes expressed in latency have been undertaken. Several different states of EBV latency have been identified i.e. different patterns of gene expression in different circumstances of latent infection. Table 1.3 illustrates the different situations and which latent genes are expressed.

1.2.5 Latency Type III

Within EBV positive lymphoblastoid cell lines (LCLs), generated *in vitro* by culturing peripheral B lymphocytes from EBV positive individuals, the whole contingent of known latency genes is expressed this includes the six EBV nuclear antigens (EBNAs) named EBNA1, 2, 3A, 3B, 3C and LP (leader protein), three latent membrane proteins (LMP) LMP 1, 2A and 2B and the two EBV infected cell RNAs otherwise known as EBERs. In addition a family of transcripts of unclear function from the *Bam*HI A region of the genome, known as BARTs are expressed (Brooks *et al.*, 1993). An alternative nomenclature where the EBNAs are numbered from 1 to 6 (respectively) is used by some. This pattern of latent gene expression, with the full array of EBV latent proteins expressed, is described as latency type III (Rickinson & Kieff, 1996). As well as characteristic of LCLs this form of expression occurs in infectious mononucleosis and in EBV positive lymphomas in transplant patients (Tierney *et al.*, 1994, Laytragoon-Lewin *et al.*, 1997). EBV immortalised LCLs display high levels of B-cell activation markers (CD23, CD30, CD39 and CD70) and cellular adhesion molecules (ICAMI, LFA1 and LFA3) (Rooney *et al.*, 1986, Gregory *et al.*, 1988).

Type of Latency	EBV Genes Expressed	Circumstance Detected
I	EBNA 1 EBERs BARTs	EBV infected resting B cells in immunocompetent host (Chen *et al.*, 1999) Endemic Burkitt's Lymphoma (Rowe *et al.*, 1987)
II	EBNA1 LMP1 (some) LMP 2A/LMP 2B EBERs BARTs	NPC (Brooks *et al.*, 1992) Hodgkin's Lymphoma (Deacon *et al.*, 1993)
III	EBNA 1 EBNA 2 EBNA 3A EBNA 3B EBNA 3C EBNA LP LMP 1 LMP 2A LMP 2B EBERs BARTs	EBV infected lymphoblastoid cell lines (LCLs) Post transplant lymphoproliferative disease (PTLD) (Young *et al.*, 1989) Lymphoproliferative disease in AIDS patients (Hamilton-Dutoit *et al.*, 1993, Brink *et al.*, 1997) Infectious mononucleosis (Tierney *et al.*, 1994)

Table 1.3. The EBV latency genes and the circumstances in which they are expressed.

1.2.6 Latency Type I

Endemic Burkitt's lymphoma biopsies display EBV latency type I which is characterised by the expression of EBNA1 and the EBERs (Rowe *et al.*, 1987). The BARTs are also detected in latency type I (Brooks *et al.*, 1993). EBNA1 is required for the maintenance of the genome by ensuring equal partitioning of viral DNA to the daughter cells as cells divide. EBNA1 has a glycine-alanine repeat region which confers upon it the ability to evade degradation by the host cellular proteosome and subsequent MHCI presentation to cytotoxic T cells (Levitskaya *et al.*, 1995 & 1997). After prolonged culture of BL tumour explants *in vitro* some BL cell lines drift towards latency type III phenotype suggesting that *in vivo* host factors (immune surveillance) dictate the necessity of restricted EBV gene expression to enable persistence of the lymphoma (Rowe *et al.*, 1986).

1.2.7 Latency Type II

NPC and HD are two classic examples of latency II disease lymphomas (Deacon *et al*, 1993, Brooks *et al*, 1992 & Hamilton-Dutoit *et al*, 1993, Young *et al.*, 1988). In latency II the EBV gene expression is limited to EBNA1, LMP1 (in some cases), 2A, 2B and the BARTs (Hitt *et al.*, 1989, Gilligan *et al.*, 1991). LMP1 is thought to play a role in the development of these malignancies given that LMP-1 can induce morphological transformation in human keratinocytes (Hu *et al.*, 1993). LMP1 can transform immortal rodent fibroblast lines (Baichwal & Sugden, 1988).

In resting B cells of the persistently infected immunocompetent individual latent EBV gene expression includes only the EBERs and LMP2A (Miyashita *et al.*, 1995, Decker *et al.*, 1996, Miyashita *et al.*, 1997). LMP2A is thought to prevent the virus from reactivating by exerting negative effects on stimulatory cellular transduction pathway signalling (Miller *et al.*, 1995). As the cells are not dividing EBNA1 is not required for genome maintenance.

1.2.8 Herpesvirus Saimiri

The T lymphotrophic herpesvirus saimiri (HVS) is the prototypical gamma2herpesvirus which can be consistently isolated from the blood of the squirrel monkey (*Saimiri sciureus*) it's natural host. Infection of the squirrel monkey is asymptomatic, however in other species of New World primate and the cotton tail rabbit HVS infection results in fulminant T cell lymphomas (Wright *et al.*, 1977, Medveczky *et al*, 1989). Three strains, types A, B and C have been defined for HVS according to high variability in the leftmost 7 kilobases of the long unique region of the viral genome which are responsible for the oncogenicity of the virus (Medveczky *et al.*, 1984).

In vitro, HVS can transform human and New world monkey T cells to continuous growth (Schirm *et al*, 1984 & Biesinger *et al*, 1992), they grow independent of antigen presenting cells and antigen stimulation. Some (but not all) HVS transformed T cells require the presence of interleukin 2 (IL-2) (Szomolanyi *et al.*,

1987, Medveczky *et al.*, 1993). The T cell transforming ability of HVS has been viewed as a powerful culture system for studying T cell biology. HVS transformed T cells do not require antigen and accessory cell stimulation to grow continuously, previously unavailable features that inhibited studies of T cell function. The T cell subtypes HVS can transform *in vitro* include $CD4^+$, $CD8^+$ and γ/δ T cells (Berend *et al*, 1993 & De Carli *et al*, 1993, Yasukawa *et al*, 1995). Importantly the HVS transformed T cells retain their cytokine production profile and $CD8^+$ T cells retain their antigen specificity and cytotoxicity (Weber *et al.*, 1993, De Carli *et al*, 1993, Berend *et al*, 1993 & Mackewicz *et al*, 1997). Additional to the transforming properties of HVS, HVS has been considered as an experimental model to study gammaherpesviruses and also for use as a viral vector e.g. in human gene therapy (Stevenson *et al.*, 1999a & 2000).

1.2.9 HVS Genome

HVS has a 112.9kbp unique DNA region of low GC content (34.5%) flanked by approximately 35 noncoding 1.444kbp repeats of high GC content (70.8%) and encodes at least 76 open reading frames. Of the ORFs, 60 have counterparts in other herpesviruses, the majority of which are the conserved and semiconserved herpesvirus genes (refer to table 1.2). In addition there are gammaherpesvirus, type 2 gammaherpesvirus and HVS specific genes. HVS demonstrates that gamma2herpesviruses seem to be particularly prone to sequestering cellular genes, HVS encodes at least 15 genes with homology to cellular genes including D-type cyclins, human interleukin 8 receptor ($IL8^R$), CD59, complement control proteins, thymidylate synthase and dihydrofolate reductase, this is on the high side in comparison to other herpesviruses (Albrecht *et al.*, 1992).

HVS encodes the herpesvirus conserved glycoprotein genes displayed in table 1.2. However, ORF 51, located at the site of gp340/220 of EBV and gp150 of MHV-68 shares no significant homology with its counterparts.

1.3.1 Kaposi's Sarcoma Associated Herpesvirus

KSHV, the eighth human herpesvirus, was discovered only six years ago, following detection of portions of the genome in Kaposi's sarcoma tissue (Chang *et al.*, 1994, Moore & Chang, 1995). Intense investigation has determined KSHV to be associated with three human malignancies, Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). There are reports of detection of the KSHV genome in all the epidemiological forms of KS, which include 'classic' (presenting in elderly Mediterranean men), endemic (localised to Central Africa), human immunodeficiency virus (HIV)-associated and post-transplant. Not only does epidemiological evidence support KSHV as an aetiological cofactor of KS but also, patients treated with foscarnet (an antiviral agent known to inhibit KSHV replication) experienced regression of KS lesions (Morfeldt & Torssander, 1994). KSHV genome has been consistently detected in primary effusion lymphomas (PEL), also known as 'body-cavity-based lymphomas' (BCBL), this is a rare form of AIDS-related B-cell lymphoma which presents as malignant lymphomas in the pleural or abdominal cavity. Usually EBV and KSHV are both present in PEL (Cesarman *et al.*, 1995a & b, Gessain *et al.*, 1997) but there are cases where KSHV has been detected in the absence of EBV (Carbone *et al.*, 1996, Renne *et al.*, 1996a, Cesarman *et al.*, 1996) and of PEL sufferers that are HIV negative (Carbone *et al.*, 1996, Nador er al., 1995). KSHV is also present in a large proportion of cases of multicentric Castleman's disease, an angiolymphoproliferative lesion, particularly those associated with HIV-infection (Soulier *et al.*, 1995; Gessain *et al.*, 1996). KSHV is considered to be very closely associated with KS and PEL, but less closely with MCD. As with EBV the occurrence of KSHV associated disease is greatly enhanced in the immunosupressed e.g. AIDS patients and post transplant patients.

1.3.2 KSHV Epidemiology

Unlike many other herpesviruses KSHV does not appear to be ubiquitous i.e. it may have a more limited presence among the human population. KS occurs in a cluster of well defined populations (e.g. Mediterranean men, central African populations, AIDS sufferers and post transplant patients) (Beral, 1991a). KSHV is thought to be

predominantly sexually transmitted, epidemiological studies show a high prevalence in AIDS patients with KS (83-88%) and also in KS-negative homosexual men (35%), in comparison the KSHV seroprevalence of women with no sexual exposure and HIV negative blood donors is very low (0-1%) (Kedes *et al.*, 1996, Gao *et al.*, 1996, Blackbourn *et al.*, 1999). There is also grounds to suggest vertical transmission of KSHV occurs due to the incidence of childhood KS. KSHV is thought to be transmitted via infected mothers breast milk as childhood KS commonly presents in a distribution likely to have arisen from virus entry of the gastrointestinal mucosa (Ziegler *et al*, 1996). KSHV has been detected in saliva (Boldogh *et al.*, 1996, Koelle *et al.*, 1997, Veira *et al.*, 1997, Blackbourn *et al.*, 1998) and may be the route of horizontal KSHV transmission. The detection of seroconversion to KSHV is a useful indication of exposure to KSHV and preempts the onset of KS.

1.3.3 KSHV Tropism

KSHV displays tropism for spindle-like cells, thought to be of endothelial origin that dominate the varied cell types present in KS lesions (Staskus *et al.*, 1997, Davis *et al.*, 1997, Rainbow *et al.*, 1997). KSHV has also been detected in circulating endothelial cells, B cells, $CD8^+$ T cells, macrophages and prostatic glandular epithelium (Boshoff *et al.*, 1995, Ambroziak *et al.*, 1995, Li *et al.*, 1996; Corbellino *et al.*, 1996b, Harrington *et al.*, 1996, Staskus *et al.*, 1997; Sturzl *et al.*, 1997; Sirianni *et al.*, 1997). Like EBV, KSHV is difficult to culture *in vitro* with only very limited replication achieved in fibroblastoid cells (Foreman *et al.*, 1997, Renne *et al.*, 1996a & 1998). KSHV establishes a latent infection characterised by expression of a restricted set of viral genes and the presence of circular KSHV genome (Russo *et al.*, 1996, Renne *et al.*, 1996b). Latently infected B cell lines have been established from PEL and the peripheral blood mononuclear cells (PBMCs) of individuals suffering from PEL e.g. BCBL-1 (Cesarman *et al.*, 1995b, Arvanitkis *et al.*, 1996, Renne *et al.*, 1996a, Said *et al.*, 1996, Boshoff *et al.*, 1998). In order to study virus ultrastructure and lytic virus gene expression, lytic replication can be induced by the addition of phorbol esters and sodium butyrate (Miller *et al.*, 1996 & 1997). As with EBV, some low level spontaneous lytic replication does occur in the KSHV infected B lymphoma cell lines. Although difficult, KSHV has been cultured

directly from KS lesions (Foreman *et al.*, 1997) and saliva (Viera *et al.*, 1997). The range of cells that will support productive infection of HVS and KSHV *in vitro* is very limited (Renne *et al.*, 1998) there is essentially no information on what these primate rhadinoviruses may use as a receptor for entry to cells.

1.3.4 KSHV Genome

Initially sequence analysis of a large genomic region (21kb) containing blocks of structural genes was done and showed that KSHV was most closely related to HVS (Moore *et al.*, 1996). Subsequently full sequence analysis was performed on KSHV from a PEL cell line (BC-1) (Russo *et al.*, 1996). The KSHV genome consists of a 140.5kbp long unique region (LUR) flanked by multiple GC rich (85%) terminal repeats of 801bp units. There are five internal repeat regions within the LUR. At least 81 ORFs have been identified, of these 66 have homology to ORFs of HVS. These HVS homologous genes include the full complement of herpesvirus conserved and semi-conserved genes (see table 1.2) and further gammaherpesvirus and rhadinovirus specific genes. KSHV has a number of KSHV specific genes and overall, in comparison to other herpesviruses, has a particularly extensive number of genes sharing homology with mammalian counterparts e.g. bcl-2, cyclin D, macrophage inflammatory protein (vMIP), OX-2 an NCAM-like adhesion molecule (vOX), IL-6 and interferon regulatory factors (vIRF).

The existence of open reading frame K8.1 was confirmed experimentally (Chandran *et al.*, 1998), it was not identified following initial sequence analysis (Russo *et al.*, 1996). K8.1 encodes a small (37kDa) virion surface membrane glycoprotein, a positional and possibly functional homologue of gp340/220 of EBV and gp150 of MHV-68 (described in more detail in section 1.7.8).

1.3.5 Animal Models

Analysis of the biology and pathogenicity of EBV and KSHV has been limited by their inherent species specificity and restricted growth *in vitro*. Before the analysis of MHV-68, animal models for the study of gammaherpesviruses were limited to

HVS infection of primates or rabbits and EBV infection of rhesus macaques (Moghaddam *et al.*, 1997) common marmosets (*Callithrix jacchus*) and cotton top tamarins (*Saguinus oedipus oedipus*) (Finerty *et al.*, 1992). Following EBV infection common marmosets suffer a poorly defined IM like syndrome (Emini *et al.*, 1986, Wedderburn *et al.*, 1984) however they have not been suitable to study secondary diseases associated with EBV. Cottontop tamarins receiving large doses of EBV experience lethal lymphomas (Cleary *et al.*, 1985). Persistent infection is less clear, low level latent infection of the tamarin has been reported however this was achieved when animals were immunised with an EBV vaccine prior to EBV challenge (Niedobitek *et al.*, 1994). Chimpanzees can be infected by human EBV and this has occurred by unintentional transfer from humans to chimps in play units however their limited availability impedes their use (Levine *et al.*, 1980). Infection of rabbits with an EBV related herpesvirus isolated from the baboon (*Macaca arctoides*) has also been proposed as an animal model for the investigation of antiviral drugs and vaccines against EBV (Wutzler *et al.*, 1995, Hayashi & Akagi, 2000). A 'humanised' severely combined immunodeficient (SCID)/hu-PBL-SCID mouse model has been used in development of therapies to treat EBV induced malignancies (Johannessen & Crawford, 1999, Mosier *et al*, 1992, Buchsbaum *et al*, 1996). The SCID mice lack mature B or T cells but have parts of immune function reconstituted, at least transiently, by infusions of human lymphocytes. The use of humanised SCID mice suffers from the inherent cross species differences that may hinder the outcome and interpretation of results.

MHV-68 closely parallels the genetic and pathobiological characteristics of EBV however, MHV-68 replicates relatively well *in vitro* and so is an invaluable tool to study gammaherpesvirus gene expression during the lytic virus cycle. Furthermore, because MHV-68 is a pathogen of mice it is also a good model to study the roles of specific gammaherpesvirus genes *in vivo* and the immune response employed by the host to control gammaherpesvirus infections. Use of MHV-68 as a model system may facilitate the development of prevention and treatment of gammaherpevirus infections and diseases.

1.3.6 Murine Gammaherpesvirus 68

The discovery of murine gammaherpesvirus 68, (MHV-68) was reported in 1980, following isolation from the brain of a bank vole, *Clethrionomys glareolus*, in Slovakia (Blaskovic *et al.*, 1980). Based on genomic analysis, revealing similarities in structure, content and organisation, MHV-68 was classified a gammaherpesvirus (Efstathiou *et al.*, 1990a & b, Mackett *et al.*, 1997). MHV-68 has been fully sequenced and approximately 80 open reading frames (ORFs) have been determined for the genome which consists of 118.2kbp unique DNA flanked by variable copies of 1.23kb repeat sequences (Virgin *et al.*, 1997. Davison, unpublished observations). The GC content of the unique region and the terminal repeats is 46% and 78% respectively. The GC content of the unique region is low compared to EBV (GC content 60%) and consistent with other gamma 2 herpesviruses unique region e.g HVS and KSHV which have a GC content of 34.5% and 53.5% respectively (Albrecht *et al.*, 1992, Russo *et al.*, 1996). The unique region of the genome has large blocks of conserved genes, with high colinearity to equivalent gene blocks of the genomes of KSHV, HVS and EBV, interspersed by regions containing virus specific ORFs. As many as 63 ORFs with homology to those of HVS and KSHV have been identified. The overall identity of MHV-68 ORFs to their homologues in the other gammaherpesviruses indicates that MHV-68 is more closely related to HVS and KSHV than to EBV and has been therefore been classified a gamma2herpesvirus (Virgin *et al.*, 1997). Refer to figure 1.3 showing the genomic organisation of MHV-68.

The conserved herpesvirus family genes present include structural proteins such as capsid proteins and the glycoproteins gB, gH, gM and gL, genes encoding proteins for virus assembly e.g. packaging proteins, proteins involved in nucleic acid metabolism; uracil DNA glycosylase, deoxyuracil triphoshatase and ribonucleotide reductase large and proteins involved in genome replication e.g. DNA polymerase and helicase-primase. The semi-conserved herpesvirus genes encoded by MHV-68 include thymidine kinase and the small subunit of ribonucleotide reductase. In summary, all the conserved and semiconserved genes displayed for

gammaherpesviruses in table 1.2 are present in MHV-68 except for a homologue to ORF41 of HVS.

On following page:

Figure 1.3 Organisation of the MHV-68 genome. The unique region of the genome is represented by a single black line with co-ordinates marked off in kilobase pairs. Terminal repeat (TR) and internal repeat (IR) regions are marked by green boxes. The open reading frames (ORFs) are indicated by arrows, the direction of the arrow indicates the direction of transcription. ORFs which are unique to MHV-68 are indicated by yellow arrows. The gene number is shown except where the function of the gene is known. Functions are abbreviated as follows: *crp*; complement regulatory protein; *gB*, glycoprotein B; K3, homologue of the immediate early gene of HHV-8; *TK*, thymidine kinase; *gH*, glycoprotein H; *gM*, glycoprotein M; *ung*, uracil DNA glycosylase; *gL*, glycoprotein L; *rta*, R (immediate-early) transcriptional transactivator; *gp150*, glycoprotein with homology to gp340/220; *dut*, dUTPase; *mta*, M (immediate-early) transcriptional transactivator, *rr2*, small subunit ribonucleotide reductase; *rr1*, large subunit ribonucleotide reductase; *cyc D*, homologue of mammalian cyclin D; *bcl-2*, homologue of mammalian bcl-2; *IL-8^R*, homologue of mammalian interleukin-8 receptor. The red ring marks the position of gp150, ORFs encoding glycoproteins are denoted by blue arrows. Adapted from Stewart *et al.*, 1999c.

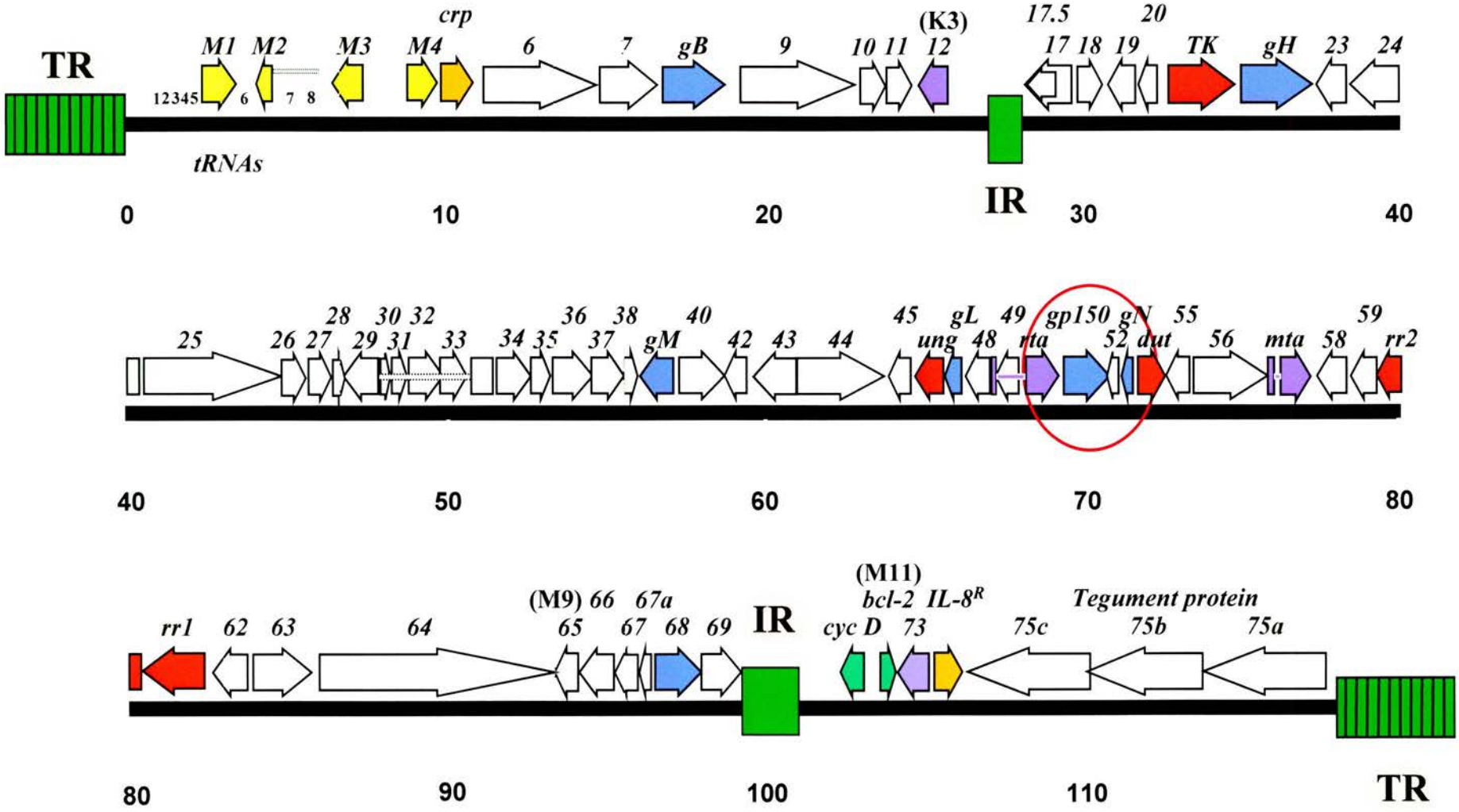


Figure 1.3. The MHV-68 genome

ORF 51, also known as M7, is specific to MHV-68 and encodes glycoprotein 150 (gp150). As mentioned earlier sequence comparisons have shown gp150 to share significant homology with gp340/220 and there is a potential homologue at the same location of KSHV. Gp150 is described in detail in section 1.7.7.

Overall the MHV-68 genes associated with lytic virus replication are well conserved however those involved in latency and transformation seem to have diverged and to be encoded by virus specific genes. There are no sequence homologues of the EBV EBNA and LMP genes or the transforming genes of HVS. At the point of individual ORFs there is great variability in the level of viral and cellular homology, there are several ORFs such as those encoding bcl-2 and TK, for which the MHV-68 encoded protein is more closely related to the EBV gene product than to that of HVS and KSHV (Virgin *et al.*, 1997).

1.3.7 MHV-68 Latency

Gene expression during latent MHV-68 infection is not clear, the questioned sensitivity of mRNA detection systems hinder these type of analyses. However, it is clear that MHV-68 does not encode obvious homologues of the EBV, KSHV and HVS tumour- or latency-associated genes (Virgin *et al.*, 1997).

1.3.8 Pathogenesis of MHV-68

Following intranasal (i.n.) inoculation of MHV-68 into mice, three to four weeks of age, an acute infection of the lung develops. The acute infection of the lung lasts for approximately ten days and is associated with inflammatory infiltrates. Infectious virus titre in the lung peaks around 5 days post infection and is cleared by day 14. Viral antigen is predominantly located in lung epithelial and mononuclear cells (Sunil-Chandra *et al.*, 1992a). Virus spreads from the lung to the spleen (and sometimes is detectable in other organs such as the heart, kidney, adrenal glands) but productive viral replication is limited at this site. The acute infection gives way to the establishment of a life long persistent/latent infection. Viral latency is predominantly located to the spleen in which the B lymphocytes have been shown to specifically harbour the latent virus (Sunil-Chandra *et al.*,1992b & Usherwood *et al*,

1996b). Latent virus first becomes detectable in the spleen at seven days post infection. The latent virus titre steadily increases peaking fifteen days post infection. Peak latent virus titre is associated with a marked $CD4^+$ T cell driven splenomegaly (Ehtisham *et al.*,1993 & Usherwood *et al.*, 1996a) a phenomenon similar to the Epstein-Barr virus induced infectious mononucleosis. During splenomegaly B cell, $CD4^+$ T cell and $CD8^+$ T cell numbers all increase by 2 – 3 fold. Of the $CD8^+$ T cells there is a predominance expressing a Vβ4 positive phenotype which suggests the presence of a superantigen expressed by MHV-68 (Tripp *et al.*, 1997). After the peak, latent virus titres decrease to a low but detectable level, never being completely eliminated. The lung has also proven to be a major site of MHV-68 latency. Viable episomal DNA has been detected in the lungs of MHV-68 infected μMT mice, lacking B cells, and intact mice, with and without antiviral drug treatment inhibiting productive viral replication, months post infection (Stewart *et al.*, 1998, Usherwood *et al.*, 1996b).

$CD8^+$ T cells play a critical role in control of the acute infection of the lung. Intranasal inoculation of MHV-68 into mice depleted of $CD8^+$ T cells gives rise to a lethal infection. Productive virus titres were highly elevated in both the lungs and spleens of the $CD8^+$ T cell depleted mice. The $CD4^+$ T cell depleted mice however, were largely unaffected, though did fail to develop a splenomegaly (Ehtisham *et al.*, 1993). Not only are $CD4^+$ T cells essential for the development of splenomegaly, they are also required for the elevated levels of splenic viral latency seen at day fifteen post infection (Usherwood *et al.*, 1996a). Humoral responses do not become detectable until after the acute infection has been resolved but once established remain high throughout the animals life (Stevenson *et al.*, 1998). The mechanism by which $CD8^+$ T cells effect killing has yet to be established. MHV-68 infection and response of mice deficient in perforin, one of the main methods of killing used by $CD8^+$ T cells and natural killer cells, does not appear to differ significantly from MHV-68 infection in wild type mice (Usherwood *et al.*, 1997).

1.3.9 MHV-68 Persistence

As outlined, the spleen and lungs are sites of viral persistence in a latent form. Viral latency is characterised by limited viral gene expression and viral genome present in a closed circular form i.e. episomal DNA. Current evidence for EBV suggests that latently infected B lymphocytes are an essential feature for EBV persistence. There are conflicting theories as to whether epithelial cells are secondary or not for the maintenance of EBV in the host.

The bone marrow is considered the major site of EBV latency. The suggested theory is that mucosa is not essential for EBV persistence and EBV is only present in the mucosa due to reseeding form the lymphoid compartment. Little evidence exists for the presence of latent EBV in the mucosal epithelium, however low levels may have been missed and it is difficult to examine this question in humans due to difficulties in obtaining biopsy material. MHV-68 provides an opportunity to examine possible sites of latency undetected previously. The revelation of MHV-68 latently occupying the lungs long term, highlights the possible association of gammaherpesviruses with lung pathologies. EBV has been detected in cells exfoliated from the lung (Lung *et al.*, 1985) and in the lung tissue of cryptogenic fibrosing alveolitis (CFA) (Egan *et al.*, 1995, Stewart *et al.*, 1999b) suggesting that the lung may be a reservoir of EBV and sometimes associated with lung pathology. Fibrosis of the lungs has been detected in interferon gamma receptor knock out mice infected with MHV-68 (E. Ebrahini, unpublished observations). Lung arteritis has also been attributed to MHV-68 when observed in mice following long term MHV-68 infection (Weck *et al.*, 1997).

1.4.1 Cytokine Profile

In response to intranasal infection of MHV-68 into mice high levels of interferon gamma (IFN-γ) and interleukin 6 (IL-6) are produced in the lymphoid tissues, levels peaking at day ten post i.n. infection. Interleukin 2 (IL-2) and interlekin 10 (IL-10) are present but at much lower levels (Sarawar *et al.*, 1996). IFN-γ does not appear essential for recovery from acute infection. In mice deficient of IFN-γ there is little

difference in the phenotype of MHV-68 infection compared to wild type mice (Sarawar *et al.*, 1997). However, following intranasal inoculation of MHV-68 into IFN-γ receptor knockout mice, the spleens showed atrophy with a reduction in splenic B cells, $CD4^+$ and $CD8^+$ T cells (Dutia *et al.*, 1997). Alpha and beta interferon (IFN-α and IFN-β respectively) appear to be of great importance in the control of acute MHV-68 infection. Shortly after intranasal inoculation of MHV-68, transgenic mice negative for IFN-α/β receptors suffer from a lethal infection (Dutia *et al.*, 1999b).

1.4.2 Lymphoproliferative Disease

MHV-68 is associated with the development of lymphoproliferative disease in mice during long term infection. 9% of inbred mice, infected intranasally with MHV-68, developed lymphomas over a period between nine months to three years post infection. Fifty percent of these mice displayed high grade lymphomas. In mice immunosuppressed by treatment with cyclosporin A before and during infection the incidence of lymphomas increased to 60%. The lymphomas were associated with lymphoid, liver, lung and kidney tissue. The lymphomas consist of B cells, predominantly light chain restricted, suggesting a clonal origin, and CD3 positive T cells. No lytic antigen positive cells were detected, however cells positive for viral DNA were detected in and at the periphery of lymphomas (Sunil-Chandra *et al.*, 1994). The S11 cell line which is positive for episomal MHV68 genome was generated from an infected B cell lymphoma (Usherwood *et al.*, 1996c).

1.4.3 MHV-68 Cellular Tropism & Latency

Unlike HVS, MHV-68 is B cell tropic with respect to latency. MHV-68 establishes latency in splenic B cells *in vivo* (Sunil-Chandra *et al.* 1992b, Usherwood *et al.*, 1996b). *In situ* hybridization and immunostaining has indicated epithelial cells and monocytes in the lung tissue harbour latent virus. There is also evidence, resulting from intra peritoneal infection, demonstrating MHV-68 can establish latency in peritoneal macrophages (Weck *et al.*, 1999). Furthermore, MHV-68 will also persistently infect transformed cell lines of B-cell origin, such as the NS0 mouse myeloma cell line and the S11 B cell lymphoma line, but not those of T cell origin.

Persistently infected NS0 B cells were shown to contain episomal DNA (Sunil-Chandra *et al.*, 1993).

As yet, the receptor MHV-68 uses to attach to cells is unknown. Unlike EBV, MHV-68 can infect and replicate productively in many different cell types *in vitro*, including epithelial and fibroblastoid cell lines originating from rodents and primates (Rajcani *et al.*, 1985), suggesting it may use a ubiquitous cellular receptor to enter cells. Following gastric instillation of MHV-68 gastrointestinal epithelial cells have been shown to undergo lytic MHV-68 infection (Peacock & Bost, 2000). In an investigation into the interaction of MHV-68 with murine splenocytes $CD43^+$ cells (T lymphocytes, macrophages and activated B cells) were shown to be readily susceptible to MHV-68 infection in addition to resting B cells (Dutia *et al.*, 1999a).

1.4.4 Glycoproteins

Glycoproteins consist of proteins that have been modified by the covalent attachment of an oligosaccharide group or groups to specific amino acids. In general eukaryotic cells produce glycoproteins with N- and/or O-linked glycosylation. N-linked glycosylation refers to oligosaccharides that are covalently attached to the amino group of asparagine residues present in the consensus N-linked glycosylation motif of Asn-X- Ser/Thr. O-linked glycosylation describes oligosaccharides that are linked via the hydroxyl group of a serine or threonine residue.

1.4.5 Glycoproteins - General Location & Roles

Glycoproteins are abundant in plasma membranes of eukaryotic cells, they perform assorted functions such as acting as receptors, ligands, ion channels, intercellular adhesion molecules etc. Many secreted proteins are glycoproteins including hormones, cytokines and immunoglobulins. The hydrophillic nature of the oligosaccharide chains increase the solubility of glycoproteins, a desirable property for proteins exposed to the extracellular environment. The sugar component of membrane glycoproteins is mainly attached to the extracellular domain of the protein. Most enveloped animal viruses, including all herpesviruses, encode genes for glycoproteins. With respect to herpesvirus replication glycoproteins can have

roles in attachment and entry into cells, virion structure, egress, cell to cell fusion, receptor formation and immune modulation (Vanderplasschen *et al.*, 1993, Foster *et al.*, 1999, Haddad *et al.*, 1989, Johnson *et al.*, 1988, Tanner & Alfieri, 1999). Virus encoded glycoproteins are generated by and undergo the same modification processes as those of the cellular host (Campadelli-Fiume & Serafini-Cessi., 1985).

1.4.6 N-linked Glycosylation

1.4.7 Generation of a lipid linked-oligosaccharide precursor

Of the two main types of glycosylation the process of N-linked glycosylation has been studied the most extensively. The first step of N-linked glycosylation is the synthesis of an oligosaccharide precursor that is linked to a lipid called dolichol (Dol) (see figure 1.4). This oligosaccharide precursor is transferred *en bloc* to nascent proteins in the endoplasmic reticulum (ER). The structure of the dolichol-oligosaccharide is conserved among eukaryotes and consists of dolichol linked via a pyrophosphate linkage to an oligosaccharide comprised of 14 monosaccharide units. The monosaccharide units include N-acetyl glucosamine (GlcNAc), glucose (Glc) and mannose (Man). The monosaccharide units are present as precursors also known as monosaccharide donors in the cytosol, ER and Golgi. The monosaccharide donors include phosphorylated intermediates GDP-Man, UDP-GlcNAc, Dol-P-Man (Dol-P = dolichol phosphate) and Dol-P-Glc . Initial attachment of monosaccharide units to dolichol occurs in the cytosol. First GlcNAc is added to dolichol by GlcNAc-1-phosphotransferase and a second GlcNAc is added by a GlcNAc-transferase both from UDP-GlcNAc donors. Tunicamycin is an analogue of UDP-GlcNAc a reagent often used to inhibit N-linked glycosylation by blocking GlcNAc-1-phosphotransferase activity in order to determine if a protein has N-linked glycosylation. Subsequent to the attachment of the two N-acetyl glucosamine residues five mannose residues are added from GDP-Man precursors. The $Man_5GlcNAc_2$-Dol formation then flips from the cytosol to the lumen of the ER by an unknown mechanism. Four mannoses are then attached from Dol-P-Man donors. Finally, three glucose residues donated by Dol-P-Glc are attached and the precursor is ready for transfer to a protein (Kornfeld & Kornfeld, 1985).

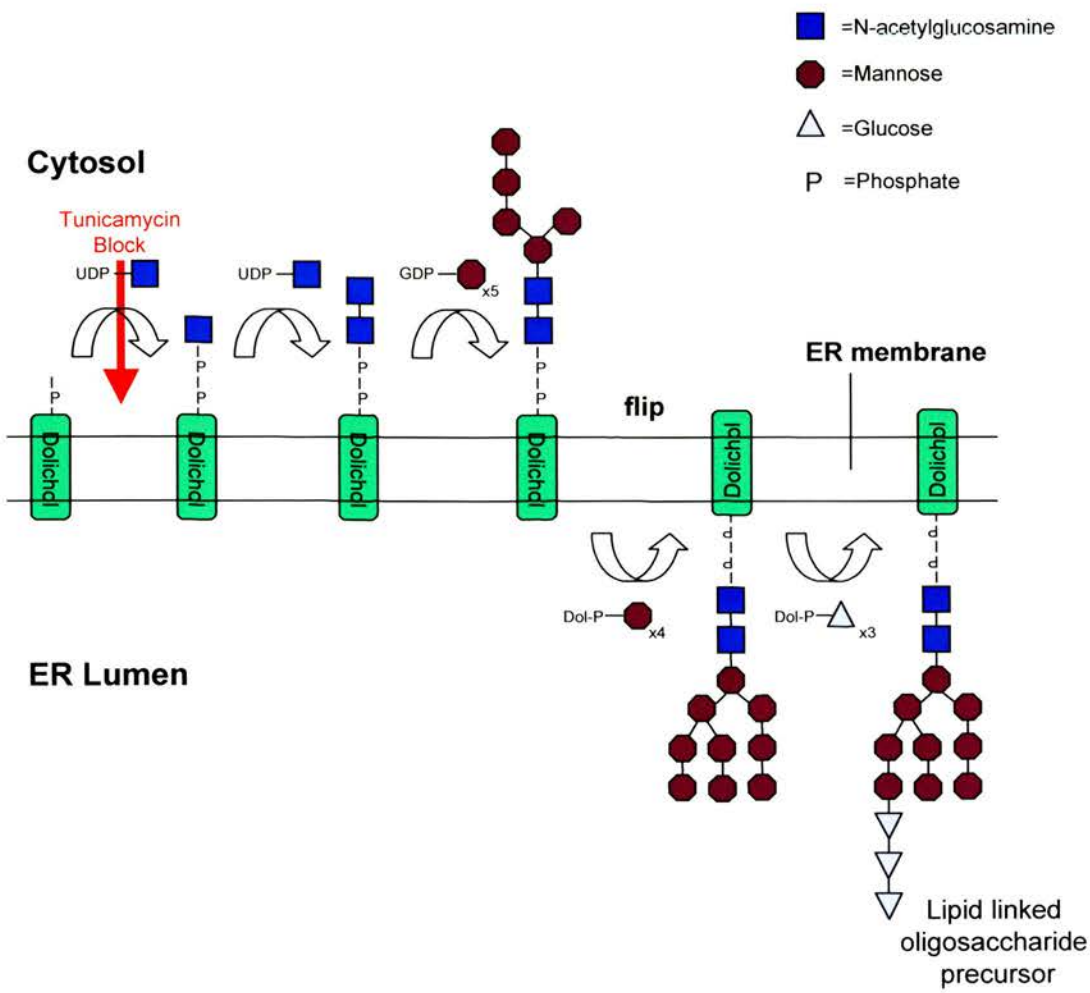


Figure 1.4. The biosynthesis of the dolichol lipid linked oligosaccharide precursor; the first step in N-linked glycosylation of proteins. Glycosyltransferases in the cytosol and endoplasmic reticulum (ER) sequentially transfer precursor sugars to the lipid dolichol. The formation of the oligosaccharide begins in the cytosol, however following the attachment of the first four mannose residues the complex flips across the ER membrane into the ER lumen where synthesis is completed. The oligosaccharide precursor is ready for transfer to nascent proteins after the attachment of the three glucose residues. Tunicamycin is an analogue of UDP-N-acetylglucosamine that blocks N-linked glycosylation by inhibiting the attachment of UDP-N-acetylglucosamine to dolichol catalysed by N-acetylglucosamine-1-phosphotransferase. Adapted from Varki *et al.*, 1999.

1.4.8 Transfer of the oligosaccharide to nascent protein

Most potential glycoproteins have a signal sequence called a signal peptide or leader sequence at the amino terminal which directs newly synthesised proteins from the ribosome to the ER lumen (von Heijne, 1986). The signal sequences found in viral glycoproteins all share a central region of uncharged hydrophobic residues often bounded by a positively charged residue on the amino terminal end. There does not appear to be any specific sequence requirement other than that the largely hydrophobic stretch be at least 11 amino acids long (von Heijne, 1986). The dolichol lipid linked oligosaccharide precursor is transferred to newly synthesised proteins rapidly as they enter the ER by a protein complex called the oligosaccharyltransferase complex (OST) which resides in the ER membrane (see figure 1.5). The OST cleaves the high energy GlcNAc-P bond and the oligosaccharide is attached via the amino group of an asparagine residue positioned in the defined N-linked glycosylation sequence of Asn-X-Ser/Thr (where X can be any amino acid except proline). Not all potential glycosylation sites are used. Studies of a rabies glycoprotein have suggested that the amino acid following the Ser/Thr residue can influence the efficiency of glycosylation (Mellquist *et al.*, 1998).

1.4.9 Cleavage of Glucose & Protein Folding

Having been attached to the nascent protein sequential processing of the oligosaccharide occurs mediated by enzymes present in the ER and Golgi. Enzymes trim and add sugars (including monosaccharides such as sialic acid, fucose and galactose in addition to the types already described in the oligosaccharide precursor) as the protein encounters them in transit through the ER. Initially glucosidase I acts to remove the terminal α1-2-linked terminal glucose. Subsequently the remaining two glucose residues are cleaved off by glucosidase II (Moremen *et al.*, 1994). The elimination of the glucose residues is associated with protein folding mechanisms.

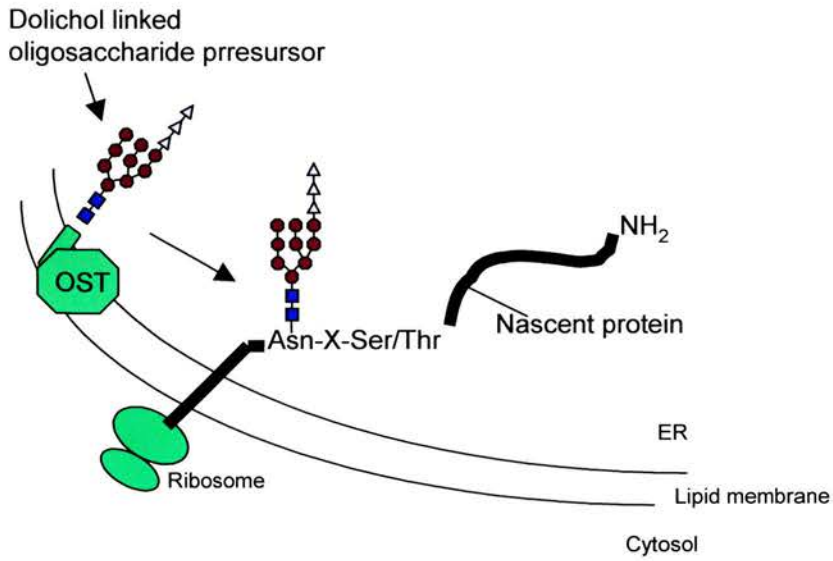


Figure 1.5. Transfer of the dolichol linked oligosaccharide precursor to a newly emerging protein in the ER. This is performed by the 'oligosaccharide transfer complex' (OST) that resides in the endoplasmic reticulum (ER) membrane. The oligosaccharide is transferred specifically to arginine in the motif Asn-X-serine/threonine (where X is any amino acid except proline) accomplishing N-linked glycosylation.

1.5.1 Importance of Glycosylation For Protein Structure Formation

Chaperones have been identified that bind to nascent proteins which appear to be involved in regulating correct protein folding. Calnexin is described as one such chaperone, this is a lectin that specifically binds to glycoproteins displaying N-linked glycans (Hammond *et al.*, 1994). The innermost glucose-linked to mannose has been found to be crucial and calnexin binds via this residue independent of the protein conformation (Herbert *et al*, 1995, Zapun *et al.*, 1997). The proposed model is that calnexin acts as a 'docking' protein retaining the polypeptide in the ER and promotes protein folding (Labriola *et al.*, 1995, Ou *et al.*, 1993). It has been reported that when calnexin is unable to associate with the glucose residue, for example in cells deficient in glucosidase I and II or when cells are treated with inhibitors of glucosidase (1-deoxynojirimycin, castanospermine), increased misfolding of glycoproteins occurs (Ora & Helenius, 1995, Saunier *et al.*, 1982). Alph-glucosyltransferase reglycosylates incorrectly folded proteins which are then either refolded or degraded (Sousa & Parodi, 1985). This shows that early in generation the presence of N-linked glycosylation influences protein structure. The mechanism for detection of incorrectly folded proteins is not fully known.

1.5.2 N-glycan Processing Pathways

The N-glycan group existing as $Man_8GlcNAc_2$-Asn following the removal of the three glucose residues is called the high mannose subtype and can now undergo further processing. There are two processing pathways that the high mannose glycans may undergo. In one pathway two of the mannose residues are phosphorylated by a series of enzyme activities which targets the glycoprotein to the lysosome. However, most glycoproteins undergo the other form of processing whereby specific α-mannosidase enzymes located in the ER and Golgi sequentially process the high mannose N-glycans (see figure 1.6) . In general the mannosidases trim off four mannose residues and the resulting $Man_5GlcNAc_2$ sugar is then subject to diverse modification in the Golgi by glycosyltransferases e.g. GlcNAcT-I and GlcNAcT-II adding sugars and glycosidases cleaving sugars e.g α-mannosidase II (Tulsiani *et al.*, 1982). A model exists where different processing enzymes are located in different cisternae of the Golgi stack and act as the glycoprotein passes

through their domain in a highly ordered sequence similar to a manufacturing assembly line (Hirschberg & Snider, 1987). The final N-linked glycan can be one of three types i.e. high mannose, hybrid or complex N-linked glycosylation. High mannose N-linked oligosaccharides have no new sugars added to them in the Golgi and are characteristic of immature glycoproteins. Most extracellular N-linked glycosylation is of the complex type where both the α3 and α6 mannose residues are substituted with GlcNAc residues. Where both substituted and unsubstitued mannose residues are present the glycan is termed a hybrid (refer to figure 1.6). Many different sugars can be attached in various degrees of branching complexity. The presence of fucose attached by the action of fucosyltransferase to the primary GlcNAc (i.e. GlcNAc linked to the asparagine residue) is a common feature of N-linked oligosaccharides.

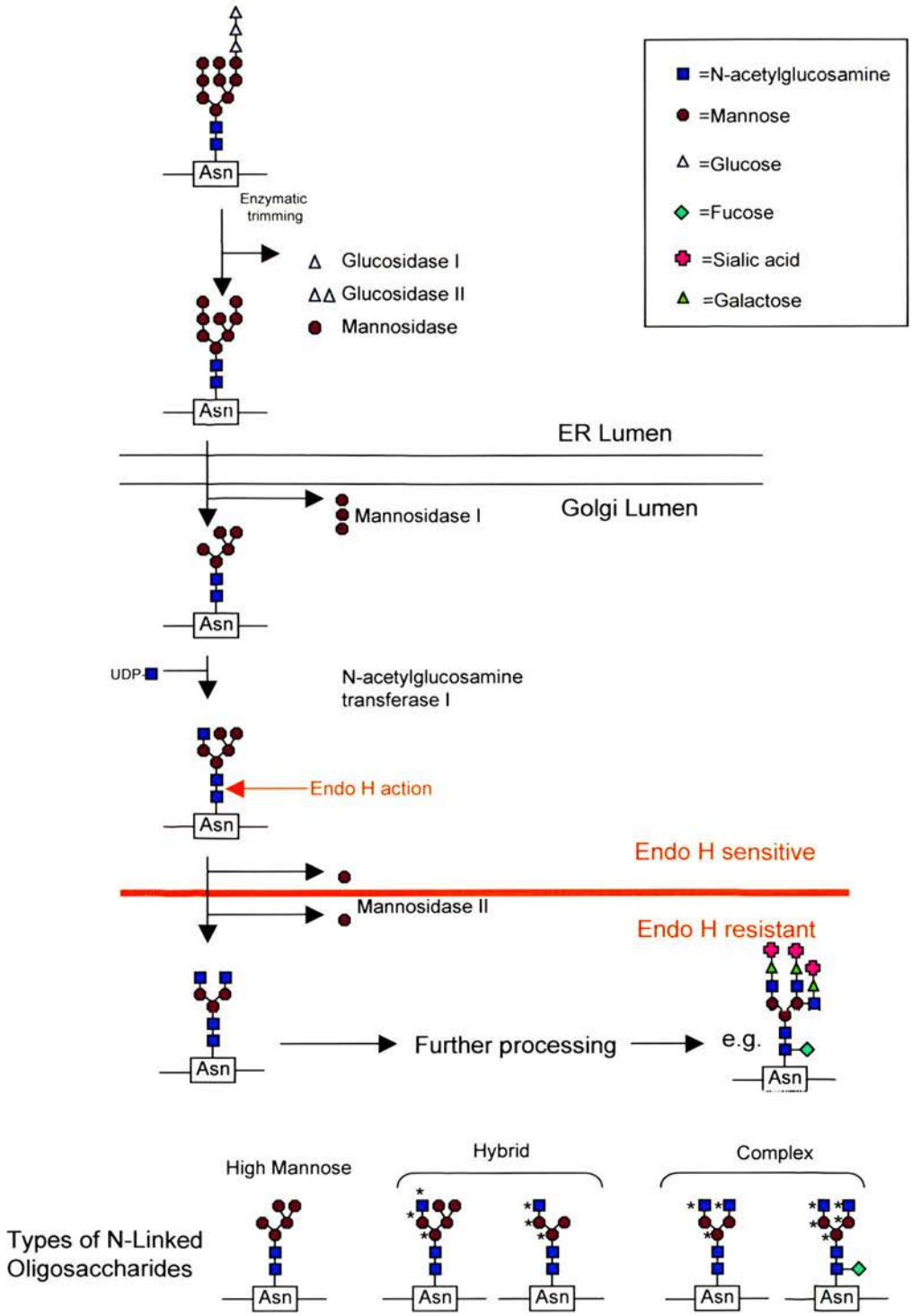


Figure 1.6. Processing of N-linked oligosaccharides in the endoplasmic reticulum (ER) and Golgi. The processing pathway occurs in a highly ordered sequence, each step is dependent on the previous one. Initially the glucose residues are cleaved off, it appears likely that only when correct protein folding has occurred is the last glucose trimmed. A mannose is removed and the protein is transferred to the Golgi where mannosidase I cleaves an additional three mannoses and N-acetylglucosamine transferase adds N-acetylglucosamine. Following the cleavage of a mannose by mannosidase II the oligosaccharide is resistant to Endo H, an enzyme which cleaves the bond between the first two N-acetylglucosamines. Endo H is a reagent commonly used to distinguish between complex and high mannose oligosaccharides. Addition and cleavage of sugars continues for complex and hybrid oligosaccharides, the star symbols indicate where branches of sugar chains can form. Adapted from Varki *et al.*, 1999 & Stryer *et al.*, 1995.

1.5.3 O-Linked Oligosaccharides

O-linked glycosylation refers to the linkage of oligosaccharides to proteins via the hydroxyl group of serine and threonine residues (small oligosaccharides can also be linked to the side chain of hydroxyproline or hydroxylysine). O-linked glycans are generally shorter and more variable than N-linked oligosaccharides. In contrast to the formation of N-linked glycosylation a lipid-linked oligosaccharide precursor is not required, the first step of glycosylation involves the direct attachment of GalNAc (from UDP-GalNAc) to serine and threonine residues by GalNAc transferase (see figure 1.7). It is thought that O-linked glycosylation occurs relatively late in the biosynthetic pathway i.e. in the Golgi (Jenkins & Curling, 1994, Johnson & Spear, 1983). There appear to be at least eight different isozymes of GalNActransferase in mammalian cells that are possibly differentially expressed among different tissues and cell types (Clausen & Bennet, 1996). No consensus sequence has been identified for O-linked glycosylation. Patterns are emerging from study of sites of O-linked glycosylation for example alanine, serine and threonine are commonly found adjacent to the glycosylated residue (O'Connell *et al.*, 1992, Wang *et al.*, 1993, Wilson *et al.*, 1991). Proline residues are frequently located around the site of O-linked glycosylation which fits in well with the common presence of O-linked glycosylation at β turns in proteins, structures that proline residues promote. O-glycans tend to be less branched than N-linked glycosylation, however the two types of glycosylation do tend to have similar terminal patterns of sugar structures. The enzyme O-glycosidase cleaves O-linked glycosylation.

There are four main core subtypes of O-linked sugars determined by the specific modification to GlcNAc and attachment of secondary monosaccharides in particular linkages (e.g. β1-3 linkage etc.) by an appropriate glycosyltransferase (see figure 1.7). Core 2 linkages are the most common linkages followed by core 1 linkages (core 3 and 4 linkages occur much less). There are further types of rarer core glycosylation. The attachment of further sugars to the O-linked glycan is thought to depend on the expression levels and subcellular distributions of glycosyltransferases as with N-linked glycosylation. The default pathway for glycoproteins (with N-linked and/or O-linked glycosylation) lacking specific retention signals involves

envelopment into membranous vesicles which transport the mature glycoproteins from the Golgi to the plasma membrane (Stryer, 1995).

1.5.4 Influences on Presence and Form of Glycosylation

There are a variety of factors that determine whether a protein becomes glycosylated or not and the diversity of oligosaccharides attached to the same protein. Protein sequence, as mentioned is key in directing the site of glycan attachment where the specific N-linked glycosylation motif is required for N-linked glycosylation and serine and threonine residues are required in a, as yet, less well defined context for O-linked glycosylation. In addition, the accessibility of potential glycosylation sites is an important factor and depends on protein conformation. Potential glycosylation sites near the N terminus are more likely to be occupied, this is possibly due to the fact that this end of the protein emerges into the ER first (Gavel & von Heijne, 1990). The transport rate of the protein through the ER and Golgi and the expression and location of glycosyltransferases dictating which enzymes a growing sugar encounters first is thought to be a factor. Also, different host cell types can produce a specific protein with different glycosylation profiles. Studies of glycoproteins produced in *in vitro* cell cultures have shown that the culture environment e.g. presence of growth factors affecting glycosyltransferases, availability of sugars etc. influences the extent of glycosylation (Jenkins & Curling, 1994). So too can the growth rate of the cells i.e. activation state. The method of cell culture can affect glycosylation and is exemplified by the different glycosylation profiles of immunoglobulins produced in ascites tumours compared to hybridomas grown in serum-based media (Patel *et al.*, 1992). In this situation the content of the oligosaccharides can vary with regard to different sugar residues incorporated which appears to influence the half life of the antibodies when injected into animals (Maiorella *et al.*, 1993). This variability is of concern in the manufacture of vaccines i.e. producing a protein in different systems may result in different glycosylation and patterns of immunogenicity.

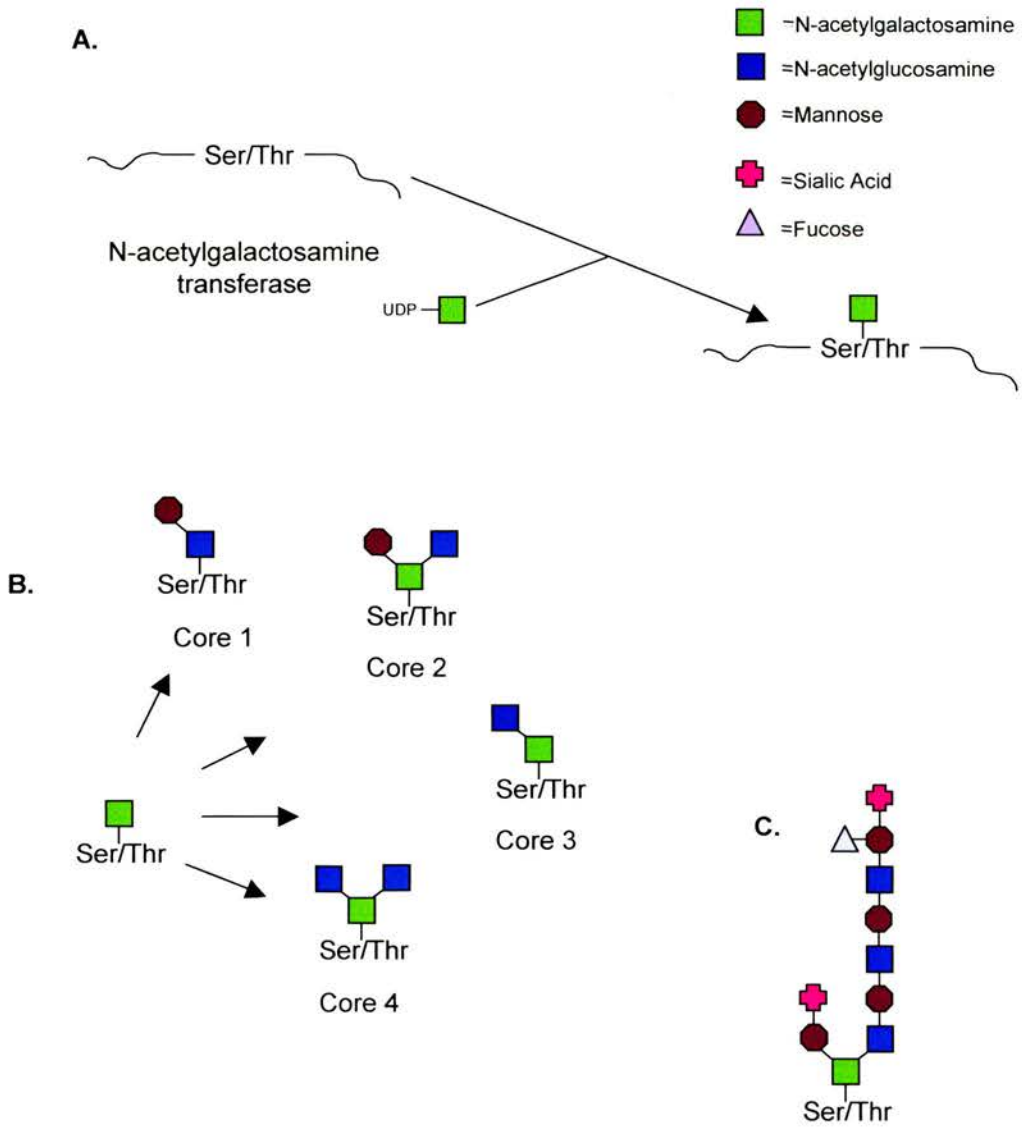


Figure 1.7. A. O-linked protein glycosylation. This begins in the Golgi and is initiated by N-acetyl galactosamine transferase attaching a N-acetylgalactosamine to serine and threonine residues of proteins. **B**. Core structures; patterns of sugar units have been identified which are formed following the attachment of the initial N-acetylgalactosamine to which further sugars are added commonly in long chains, the four main core structures are shown as an examples (Varki *et al*., 1999). **C.** An example of an O-linked oligosaccharide.

1.5.5 Function of Protein Bound Glycans

The properties of a glycoprotein commonly influenced by protein bound glycans include: solubility, stability, quarternary structure, activity, antigenicity and half-life of the protein. Sugar groups can physically shield the protein they are bound to from proteases. Glycans also contribute to directing intracellular traffic e.g. the phosphorylation of mannose early in the ER directs proteins to the lysosome (Dahms & Kornfeld, 1989). The hormone β-human chorionic gonadotrophin (β-HCG) exemplifies the importance of glycosylation for protein function, deglycosylated β-HCG is able to bind to its receptor but fails to stimulate adenylate cyclase in contrast to the glycosylated form. Glycosylation is necessary for proteolytic processing and transport of the measle glycoprotein F (Sato *et al.*, 1988). The F_2 subunit has three predicted N-linked glycosylation sites, mutation of any of these asparagines results in decreased transport to the cell surface (Alkhatib *et al.*, 1994b) and impaired proteolytic cleavage, stability and fusion capacity presumably by altering the conformation of F_2 (Alkhatib *et al.*, 1994a).

Fine tuning of the activity of growth factors has been attributed to the type and extent of their glycosylation. N-CAM, the neural cell adhesion molecule mediates homophilic binding between neuronal cells. During growth development the length of polysialic acid chains commonly found at the outermost ends of N- and O-linked oligosaccharides decrease markedly. It is thought that these sugar chains are involved in maintaining developmental plasticity by regulating intercellular interactions of neuronal cells, the lengthy oligosaccharide chains physically distancing the cells (Rutishauser *et al.*, 1985, Rutishauser, 1998). The activities of erythropoietin and plasminogen activator are modulated by the type of glycans present (Takeuchi *et al.*, 1989, Goldwasser *et al.*, 1974, Wittwer & Howard, 1990).

Glycosylation can affect antigenicity, either masking antigenic sites or being part of the epitope itself. Masking peptide epitopes with carbohydrates is a method sometimes used by viruses such as HIV for immune evasion from neutralising antibodies (Bosch *et al.*, 1994). By forming a new glycosylation site the H3 of influenza virus can escape monoclonal antibody recognition (Skehel *et al.*, 1984). Poor immunogenicity of the Ebola glycoprotein GP is thought to be due to the high

level of glycosylation it has causing GP to be less antigenic (Feldmann *et al.*, 1994, Geyer *et al.*, 1992). The N-linked glycan structure of the hormones lutropin, thyroid-stimulating hormone and pro-opiomelanocortin controls their half life. A receptor for sulfated GalNAc is located in the liver endothelium and rapidly removes these hormones specifically bearing this glycan. An example where a glycan structure functions to allow identification and clearance of a protein. Lymphocyte homing and inflammatory responses involving leukocyte intravasion are regulated by glycans that function as ligands for lectins. The selectin cell adhesion molecules provide an example where oligosaccaharide modifications act as recognition molecules (Varki *et al.*, 1992).

1.5.6 Study of Glycosylation

There are a variety of methods to determine if a viral protein is glycosylated. A preliminary indication that a protein is glycosylated is provided by the typical appearance a glycoprotein has as diffuse bands in SDS-PAGE gels due to the heterogeneity of each oligosaccharide present. The periodic acid-Schiff (PAS) reaction enables the identification of glycoproteins in gels this employs the susceptibility of glycans to periodate oxidation (Gerard, 1990). Enzymes such as endoglycosidases and exoglycosylases can be employed to determine the presence and extent of glycosylation. In practice, shifts in protein mobility may be seen in SDS-PAGE gels following the treatment of glycoproteins with enzymes such as endoglycosidase H (endo H), peptide-N-glycosidase F (PNGaseF) and endoglycosidease F2 that cleave glycan groups. Specific enzymes can indicate not only the presence of glycosylation but also the type and bonding of some sugar units present e.g. neuraminidase from *Arthobacter* hydrolyses terminal α2,3-, α2,6- and α2,8- ketosidic bonds that join sialic acid residues to oligosaccharides. Neuraminidase derived from Newcastle disease virus cleaves α2,3- and α2,8- but not α2,6- ketosidic bonds, of the two neuraminidases only the *Arthobacter* neuraminidase altered the mobility of glycoprotein N of EBV, no further shift was seen when O glycosidase was added, indicating that the majority of the sugars on the molecule were α2,6-linked sialic acid residues (Lake *et al.*, 1998). High mannose N-linked oligosaccharides are specifically sensitive to endo H which gives an indication

of the maturity and/or localisation of a protein i.e. high mannose glycoproteins tend to remain in the ER and nuclear regions and do not undergo processing to complex N-linked glycans in the Golgi and localise to the plasma membrane. Changes in size and antigenic conformation of a protein when produced in the presence of inhibitors of N-linked glycosylation such as tunicamycin is a tool used to identify glycoproteins e.g. as used by Li *et al.*, 1999 in analysis of the KSHV membrane glycoprotein K8.1. Chemical treatments e.g. hydrogen fluoride, β-elimination (alkaline borohydride treatment – under carefully controlled conditions cleaves only O-linked glycans) and hydrazinolysis (Ashford, 1992), are available that effect complete removal of N- and O-linked oligosaccharides. Lectins, which are proteins that bind carbohydrates, and glycan specific antibodies enable detection of oligosaccharide groups. Western blots can be overlayed with lectin to identify glycoproteins. Metabolic radioactive labelling using radioactive sugar precursors such as [^{3}H] or [^{14}C] labelled glucosamine is another method to detect glycosylated proteins. Other strategies to study glycoproteins include genetic manipulation of glycosylation i.e. site directed mutagenesis of potential glycosylation sites. Cell free *in vitro* synthesis of proteins and comparison with the native form can give indications about the contribution of post translational modifications which do not occur in the cell free system e.g. glycosylation to protein size, structure and function. Expression of the gene encoding the protein in alternative hosts to mammalian cells such as yeast and bacteria where glycosylation is different or does not occur also provides opportunity to assess the extent and structural/functional importance of glycosylation to a protein.

1.5.7 Contribution of Oligosaccharides to Protein Function

Many proteins produced by recombinant DNA technology and/or following expression in a heterologous host where they carry no or altered glycosylation do not appear to be affected in their function. Human derived interferon gamma produced in *E. coli* has full antiviral and antiproliferaive activity *in vitro* (Rinderknecht *et al.*, 1984). The exact nature of the carbohydrates added may not always be important, for example baculovirus-expressed haemagglutinin glycoprotein of measles virus functions in haemagglutination and fusion (Vialard *et al.*, 1990). In the absence of glycosylation glycoprotein D of herpes simplex virus is still able to function as a

binding protein (Sodora *et al.*,1991a & b). Some but not all N-linked glycosylation of glycoprotein gp120 of human immunodeficiency virus (HIV) is essential for virus replication (Lee *et al.*, 1993). The consequences of altered glycosylation are very unpredictable and can range from no effect to complete loss of function. Absence of glycosylation may have an indirect effect on function due to conformational changes in the protein.

1.5.8 In Depth Analysis of Glycoprotein Structure

The study of the exact make up of attached oligosaccharides is difficult due to the complex nature of their structure which commonly branch and have various modifications made from the initial backbone attached in the ER. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are used along with chromatography based separation to analyse the sequence of oligosaccharides. Mass spectrometery involves the determination of mass and composition by ionisation of the oligosaccharide, the resulting particles are accelerated (put under vacuum) and subject to a magnetic field, the different ions present are differentiated by their different deflection patterns i.e. according to their mass to charge ratio. NMR is based on the fact that electrons in a molecule shield the nucleus to some extent from electromagnetic radiation causing different atoms to absorb at slightly different frequencies.

The overall structure of a glycoprotein can be determined by X ray crystallography. For this the protein must be soluble and readily crystallize, high concentrations of protein are necessary. X-ray crystallography has been used extensively for elucidation of the structure of the glycoprotein haemagglutinin of influenza (Weis *et al.*, 1990, Wiley *et al.*, 1981, Wilson, 1981).

1.5.9 Study Of Viral Glycoprotein Function

Following identification of a viral glycoprotein and basic structural analysis e.g. confirming size, presence of glycosylation, localisation, time and level of expression functional studies are often undertaken. Data from basic analysis of a glycoprotein can provide initial insights into the potential role of a glycoprotein for example – if a

glycoprotein is located on the virion membrane surface it may be involved directly in initial infection of cells i.e. attachment and entry whereas a glycoprotein that is not represented on the viral membrane but resides in the nuclear membrane and ER of infected cells is likely to be involved in other processes such as virion formation.

The generation and isolation of mutant viruses that are unable to express a specific glycoprotein is a common method used to elucidate the function of the glycoprotein in virus replication. The principal method in the determination of the function of HSV glycoproteins described in sections 1.6.9-1.7.1 has been the study of mutant and recombinant viruses deficient in specific glycoproteins. In addition, binding studies of the purified glycoprotein and the generation of recombinant viruses expressing a foreign glycoprotein to see if the tropism of the virus is altered are done to determine glycoprotein function. For the study of glycoproteins of particularly hazardous viruses such as HIV and Ebola pseudotyped viruses have been employed where for example vesicular stomatis virus (VSV) or Moloney leukemia virus are engineered to express HIV and Ebola glycoproteins respectively (Pauza, 1991, Yang *et al.*, 1998). Expression of the glycoprotein of interest in cells independent of other viral proteins can provide information on whether the glycoprotein requires other viral proteins to localise and function correctly. The effect of antibodies against the glycoprotein of interest on virus replication can provide indications of whether the glycoprotein is involved in binding and/or entry of the virus into target cells.

1.6.1 Viral Membrane Proteins – Structural Classes

The majority of herpesvirus glycoproteins belong to the type I group of membrane glycoproteins. Type I proteins have a single transmembrane spanning domain usually comprised of 18-27 primarily hydrophobic amino acid residues, the bulk of the protein is extracellular with a relatively small cytoplasmic region. The N terminus of the protein is extracellular with a cytoplasmic carboxy terminus. Type II viral membrane proteins are orientated in the opposite direction whereby the carboxy terminus is extracellular, neuraminidase of influenza is a type II glycoprotein (Varghese *et al.*, 1988). Type III glycoproteins, such as gM of pseudorabies virus

(Dijkstra *et al.*, 1996) have multiple membrane spanning domains with both the amino and carboxy terminus located in the cytoplasm. There are some exceptions such as gL of HSV-1 which is entirely extracellular and is anchored to the virion membrane by forming a complex with gH (Hutchinson *et al.*, 1992).

1.6.2 Virus-Receptor Interactions

There are numerous examples of enveloped viruses that employ virally encoded glycoproteins to bind to cellular receptors which facilitate virion attachment to host cells. For some viruses a single membrane glycoprotein protein is responsible for both receptor binding and membrane fusion e.g. rhabdoviruses, whereas others such as the herpesviruses express multiple different glycoproteins to effect cell entry.

1.6.3 Influenza – Haemagglutinin

The glycoprotein of influenza called haemagglutinin (HA) has been studied in great depth and provides a good example of common glycoprotein roles; functioning in attachment and fusion. HA facilitates the attachment of influenza to cells by binding sialic acid residues linked to cellular membrane proteins. HA is a type I integral membrane glycoprotein it has an ectodomain of 512 amino acids, a transmembrane domain of 27 amino acids, and a 10 amino acid cytoplasmic tail (Verhoeyen *et al.*, 1980) which forms a homotrimer of non-covalently linked monomers (Wiley & Skehel, 1977 & 1987, Wilson *et al.*, 1981). X ray crystallography has been used to determine the 3D structure of the HA trimer (Weis *et al.*, 1990, Wiley *et al.*, 1981, Wilson *et al.*, 1981). The three repeated HA monomers form a rod like structure with a knob at the end which protrudes from the viral membrane. Alpha helices form the spike and the knob is mainly composed of beta sheets. The haemagglutinin has three sialic acid binding sites at the knob region. The interaction between influenza HA and sialic acid is considered weak but stable virus attachment is enabled due to the multiple number of interactions. HA mediates fusion of the membrane of the endocytosed virus particles with the endosomal membrane.

HA is a good example where structure of a glycoprotein is closely linked with function. Three distinct forms of haemagglutinin have been identified. The HA0 form, as it is known, is as the molecule exists on the surface of the virus prior to infection. The HA form ready for fusion results from protease cleavage at a site near the base of the spike at a particular arginine residue. The cleavage results in two polypeptide chains – HA_1(47kDa) and HA_2 (29kDa) that are linked by a disulphide bond. Cleavage is essential for infectivity and is directed by the existence of a specific cleavage signal of about 16 amino acids near the extracellular base of the HA protein. The third form of haemagglutinin, fusogenic HA, occurs at acidic pH i.e. approximately pH 5 (Stegmann *et al.*, 1987). The transition of the glycoprotein to the fusogenic form is produced by a conformational change in the protein that exposes previously concealed amino acid domains – fusion peptides. The influenza haemagglutinin has an uncharged fusion peptide sequence of which other viral fusion glycoproteins have analogous peptides e.g. those of Sendai virus, respiratory syncytial virus (RSV), HIV gp41 and the Ebola glycoprotein (Lamb *et al.*, 1999).

1.6.4 Influenza - Neuraminidase

Influenza also encodes a second virion membrane glycoprotein called neuraminidase. Neuraminidase (NA) is a homotetrameric glycoprotein(~220kDa) composed of four 453 amino acid proteins with cytoplasmic N termini (class II membrane proteins). NA functions to remove sialic acid from the surface of cells (catalysing the cleavage of the αketosidic linkage between the sialic acid and an adjacent D-galactose or D-galactosamine) (Palese *et al.*, 1974). By electron microscopy the complex appears mushroom shaped with a stalk and head. The tetrameric complex of neuraminidase has four active sites located at the head of the mushroom structure (Lentz *et al.*, 1987, Colman *et al.*, 1983). In addition to influenza, HIV, Ebola, Sendai virus also have glycoproteins that function to cleave receptors on the cell surface on the way out of the cell to prevent the newly formed virions from sticking to the cell they are leaving.

1.6.5 HIV – Env Proteins Interaction with CD4 & Coreceptors

For retroviruses the best studied virus-receptor interaction is that of HIV with the T cell surface molecule CD4. CD4 is a member of the immunoglobulin superfamily which functions as a coreceptor in the antigen and class II MHC-dependent interactions that initiates T cell activation, it is also expressed on macrophages, monocytes and dendritic cells. HIV has two envelope glycoproteins gp41 and gp120 which are formed by the cleavage (performed by a cellular protease) of a precursor called gp160. Gp120 binds to CD4 on the target cell surface to initiate infection, it is a good example of a glycoprotein determining viral tropism (Dalgleish *et al.*, 1984, McDougal *et al.*, 1991). Distinct but overlapping binding sites on gp120 have been identified for macrophages and T cells (Shioda *et al.*, 1991). Gp120 has five loops formed by disulphide bonds of highly variable domains called V1-V5. The V3 loop domain governs several properties of the virus i.e. cell tropism and cell entry efficiency. Variations in the V3 loop correlate with differences of tropism of HIV isolates for macrophages and T cells (Fouchier *et al.*, 1992, Ebenbichler *et al.*, 1993, Milich *et al.*, 1993, Shioda *et al.*, 1992).

The gp120-CD4 interaction results in a conformational change of gp41 exposing it's hydrophobic domains, required for membrane fusion, to the cell surface. Fusion of the viral and plasma membrane follows in a pH independent manner. CD4 has been identified as the receptor for HIV however in addition HIV requires a coreceptor for entry into target cells. Two main coreceptors have been identified for HIV which are G protein coupled receptors (GCRs) the chemokine receptors CXCR4 and CCR5. Coexpression of the α-chemokine receptor CXCR4 or the β-chemokine receptor CCR5 with CD4 confer susceptibility of cells to T cell tropic HIV or macrophage tropic HIV respectively (Feng *et al.*, 1996, Deng *et al.*, 1996). There is an increasing number of chemokine receptors being identified that are able to act as coreceptors for retroviruses most of which are seven transmembrane spanning GCRs (Deng *et al.*, 1997, Owen *et al.* 1998, Doranz *et al.*, 1996). Gp41 is thought to be analogous to influenza HA with a role in fusion (Freed *et al.*, 1990, Gallaher, 1987).

1.6.6 Paramyxovirus glycoproteins

Viruses of the paramyxovirus family, including Sendai virus and measles, in general have two virion envelope glycoproteins – haemmagglutinin (H) and a fusion protein (F). In some cases the haemagglutinin also displays neuraminidiase activity. H appears to have an analogous binding function to that of influenza HA. The measles H is a homodimer (Sato *et al.*, 1995) that has been shown to bind to CD46 (Dorig *et al.*, 1993, Naniche *et al.*, 1993). CD46 is a complement-regulatory protein widely distributed throughout human tissue which normally binds complement components C3b and C4b and acts as a cofactor in the proteolytic inactivation of C3b/C4b by factor I (Liszewski *et al.*, 1991).

Glycoprotein F is formed by the cleavage of a precursor F_0 (60 kDA) into two subunits F_1(41kDa) and F_2 (18kDa) these are linked by a disulphide bond. (Dorig *et al.*, 1993, Naniche *et al.*, 1993). The new amino terminus of F1 has a stretch of hydrophobic amino acid residues and it is thought that this may play and important role in fusion (Richardson *et al.*, 1986, Richardson *et al.*, 1983, Richardson *et al.*, 1980).

1.6.7 Filovirus Glycoprotein - GP

Ebola and Marburg, enveloped negative ssRNA filoviruses which cause highly lethal haemorrhagic disease in humans, have a sole glycoprotein that in a homotrimeric fashion forms spikes which protrude from the virus membrane (Elliot *et al.*, 1985). The glycoprotein known as GP exists in two forms – a transmembrane form of 120-170 kDa (Will *et al.*, 1992) and a secreted form (SGP) of 60kDa. GP is a type I transmembrane glycoprotein that mediates viral entry (Sanchez *et al.*, 1996). GP binds to endothelial cells and is thought to determine the tropism of Ebola for vascular endothelial cells (Yang *et al.*, 1998). The SGP is thought to have an immune mediated role, it may inhibit early neutrophil activation and possibly diminish any inflammatory response that might provide innate immunity to the virus. This is based on evidence that Ebola SGP has been shown to interact with neutrophils by binding CD16b which is the neutrophil-specific form of the Fc gamma receptor III (Yang *et al.*, 1998). GP is encoded by two ORFs, the primary

gene product is SGP and GP is expressed following transcriptional editing or translational frame shifting (Sanchez *et al.*, 1996). GP is highly glycosylated, N and O-linked glycosylation accounting for 1/3 to ½ of the GP mass (Feldmann *et al.*, 1994, Feldmann *et al.*, 1991).

1.6.8 Glycoproteins of HSV

Of the herpesviruses the glycoproteins of HSV-1 have been the most extensively studied. HSV-1 is known to code for at least eleven glycoproteins, gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gN (these are the abbreviated names i.e. g stands for glycoprotein) (Roizman & Sears, 1996). Four of which, gB, gH, gL, and gM, are considered core herpesvirus genes, with all sequenced herpesviruses containing homologous genes (Gompels *et al.*, 1995, Russo *et al.*, 1996, Virgin *et al.*, 1997). gB, gH, gL, gD and gK are essential for propagation of HSV-1 *in vitro* (Cai *et al.*, 1988, Roizman & Sears, 1996). Glycoproteins that are dispensable for *in vitro* replication of HSV may be very important *in vivo*, gG has been implicated in virus replication of the CNS (Weber *et al.*, 1987) and gE-gI form a hetero-oligomer Fc receptor that may act to shield virus infected cells from the host immune response (Johnson & Feenstra, 1987, Johnson *et al.*, 1988, Balan *et al.*, 1994)

1.6.9 Attachment of HSV

For HSV-1, work suggests that cell receptors for viral entry may be highly conserved or are multiple in number as many different cell types from different species are susceptible to infection *in vitro*. However, cells vary in their susceptibility to HSV infection. The sequential steps of HSV infection and glycoproteins involved in attachment and entry of target cells have been intensely studied. Initial attachment of herpes simplex viruses is mediated by gB (predominantly for HSV-2) or gC (predominantly for HSV-1) or both, these have been shown to attach to cell surface proteoglycans, namely the ubiquitous heparan sulphate (HS) (Cai *et al.*, 1988, Wudunn & Spear 1989, Spear *et al.*, 1992, Shieh *et al.*, 1992, Trybala *et al.*, 1993, Laquerre *et al.*, 1998)

Glycoprotein B exists as a homodimer and is located on the virion surface (Claesson-Welsh & Spear, 1986). Glycoprotein B negative viruses can bind to but are unable to penetrate cells (Manservigi *et al.*, 1977, Sarmiento *et al.*, 1979, Sarmiento & Spear 1979). Considered the most conserved herpesvirus glycoprotein the sequence of gB from a herpesvirus is often analysed to determine how closely the virus is related to other herpesviruses (Auerbach *et al.*, 2000).

Glycoprotein C also binds heparan sulphate but unlike gB it is dispensible for *in vitro* replication of HSV-1 (Herold *et al.*, 1991). Glycoprotein C also has an immune modulatory role, it has been shown to bind the complement component 3b and accelerate the decay of C3bBb and *in vivo* acts to mediate HSV evasion of complement attack (Fries *et al.*, 1986, Friedman *et al.*, 1984, Lubinski *et al.*, 1998)

Glycoprotein D and gH-gL also have roles in attachment/entry into the cell (Ligas & Johnson, 1988, Turner *et al.*, 1998). The interaction with HS is labile until other glycoproteins such as gD begin to participate in the entry process. By studying insertion and deletion protein variants four functional regions (I to IV) have been defined for gD that are important for virus entry (Chiang *et al.*,1994, Muggeridge *et al.*, 1990, Rux *et al.*, 1998). Glycoprotein D provides a stable attachment to cells and has been shown to bind to several different cellular receptors which have been termed herpesvirus entry mediators (Hve) and include HveA, HveB and HveC (Whitbeck *et al.*, 1997, Willis *et al.*, 1998, Rux *et al.*, 1998). HveA (formerly HVEM) is a novel member of the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family, initially identified by screening a human cDNA library for genes that could mediate HSV-1 entry into resistant cells. It is found predominantly on T cells and other cells of the immune system (Montgomery *et al.*, 1996,Whitbeck *et al.*, 1997). HSV can bind to but not enter cells negative for HveA (Montgomery *et al.*, 1996). HveC and B are poliovirus receptor related proteins (previously named PRR1 and PRR2 respectively) belonging to the immunoglobulin superfamily. Glycoprotein D binding to HveC is dependent on the native conformation of gD but independent of it's N-linked glycosylation (Whitbeck *et al.*, 1997). Complex formation between soluble gD and HveC appears to involve one or

two gD molecules for one HveC protein. (Krummenacher *et al.*, 1998). In summary, HSV entry can be mediated by two structurally unrelated types of gD receptors through related but not identical binding to gD i.e. different domains of gD are involved for binding members of the immunoglobulin superfamily compared to the novel TNF/NGF receptor (Krummenacher *et al.*, 1998). GD is essential for HSV entry, gD negative mutants can bind but are unable to penetrate target cells (Ligas & Johnson, 1988).

HveB, HveC and the 'herpesvirus Ig-like receptor' (HIgR), a splice variant of HveC, are likely to be employed by HSV-1 and -2 for entry into the human cell lines most frequently used in HSV studies (Cocchi *et al.*, 1998a). The use of these proteins *in vivo* by HSV seems likely as a relatively high level of expression of these immunoglobulin superfamily members was detected in human tissue samples from the nervous system (Cocchi *et al.*, 1998b).

1.7.1 HSV Post Attachment/Entry

Post attachment, the gH-gL complex facilitates pH independent fusion of the virion envelope to either the plasma membrane directly or the membrane of virus containing internalised vesicle, allowing entry of the virus into the cytoplasm of the cell (Forrester *et al.*, 1992, Hutchinson *et al.*, 1992). A notable characteristic of HSV-1 gH is that it requires the presence of gL to be processed correctly and locate to the plasma and virion membrane (Hutchinson *et al.*, 1992, Roop *et al.*, 1993). GL may affect the processing of gH by masking N-glycans as suggested from evidence from gH of pseudorabies, although gH localises to the virion membrane in the absence of gL the N-glycans on the pseudorabies gH are processed to a greater extent than in the presence of gL (Klupp *et al.*, 1997).

As described, the process of HSV attachment and entry occurs via multiple glycoproteins some of which form associations with each other and can exist in different oligomeric forms e.g. dimers and trimers of gD have been identified (Handler *et al.*, 1996). Individual glycoproteins function at distinct and different points in entry in a sequential, cascade-like mechanism (Fuller & Lee, 1992). The

lack of a single glycoprotein-cell surface receptor interaction reflects the normal life cycle of HSV where, in principal, it infects 2 different cell types – epithelial cells and neurons.

Following entry, uncoating occurs and the virus genome migrates to the cell nucleus. Late viral gene expression results in the production of viral glycoproteins which localise to the nuclear membrane. Virion formation and egress is thought to occur as described in section 1.1.6. Unlike some glycoproteins that are not required for the production of enveloped virus particles but are critical for virion infectivity (e.g. GD and GB), gK is not present in the virion but is essential for assembly and egress of infectious virus (Hutchinson & Johnson, 1995, Jayachandra *et al.*, 1997). GK has immature N-linked glycans and locates to the nuclear and perinuclear membranes (Hutchinson & Johnson, 1995). GK is thought to indirectly regulate the fusion of infected cells (gK negative viruses cause extensive fusion of infected cells in culture) (Hutchinson & Johnson, 1995).

1.7.2 Glycoproteins of Epstein-Barr Virus

EBV contains homologues for gB (gp110), gL (gp25), gH (gp85), gM and gN (Gong *et al.*, 1987, Heineman *et al.*, 1988, Lake *et al.*, 1998). The roles of these have been studied to varying extents. Further glycoproteins expressed by EBV include gp42 (BZLF2), gp340/220 and gp150 (BDLF3).

1.7.3 EBV Gp340/220

Of great interest is the unique glycoprotein gp340/220 possessed by EBV, the most abundant protein present on the surface of virions and a major neutralising determinant. Gp340/220 is also known as gp350/220 due to a difference in the interpretation of the resolving size of the protein observed by SDS-PAGE (i.e 10kDa). Gp340/220 can be identified as two forms either the full protein with a molecular weight of 340, 000 or a smaller version of 220,000 produced from a spliced transcript from the gp340/220 encoding gene (Beisel *et al.*, 1985). O-linked and N-linked types of glycosylation constitute about 50% of the molecular mass of gp340/220 (Morgan *et al.*, 1984b). A limited region of gp340/220 has distant

homology to HSV-1 gC (Beisel *et al.* 1985) however gp340/220 appears to be unique to EBV. Gp340/220 facilitates attachment to B cells via binding the complement receptor CD21 (also known as CR2/C3dg receptor) (Nemerow *et al.*, 1987). A nonapeptide located at the N-terminal region of gp340/220 has been identified that corresponds to a similar amino acid sequence in the complement C3dg protein, this mediates the binding to CD21 (Nemerow *et al.*, 1989). Gp340/220 may also have an immune modulatory role by promoting Fas mediated T cell apoptosis (Tanner & Alfieri, 1999). In addition to facilitating virus attachment to cells the interaction of gp340/220 to CD21 may lead to the activation of a signal transduction pathway resulting in increased CD19 tyrosine phosphorylation level which may ultimately enable efficient expression of viral genes following infection (Nemerrow *et al.*, 1989, Sinclair *et al.*, 1994). With respect to entry, the cross linking of CD21 by binding gp340/220 can result in capping (Tanner *et al*, 1987) after which the gH-gL complex is thought to induce fusion of the virion envelope to the vesicle membrane (Miller & Hutt-Fletcher, 1988). Direct fusion of EBV with the cell membrane of B lymphoblastoid cells has been observed as opposed to uptake into and fusion with a vesicle membrane seen with normal B lymphocytes (Nemerow & Cooper, 1984). Besides the direct association with a cellular receptor, EBV can gain access to cells as a complex with IgA and it has been suggested that this may be how EBV spreads to epithelial cells (Sixbey & Yao, 1992).

1.7.4 EBV Post Attachment/Entry Facilitating Glycoproteins

As is the case for gH of HSV, gL of EBV (also known as gp25) is required to enable plasma localisation of EBV gH (also known as gp85) and together they form a complex involved in virus penetration. (Yaswen *et al.*, 1993). Gp42 has been shown to complex with gH-gL and is essential for fusion but not absorbtion of EBV to B lymphocytes (Wang & Hutt-Fletcher, 1998). The extracellular domain of gp42 binds the HLA-DR beta chain (MHCII) and can inhibit antigen presentation (Spriggs *et al.*, 1996). A model has been devised where two different types of complex mediate EBV infection of epithelial cells and B cells i.e. gH-gL can enable infection of epithelial cells and gH-gL plus gp42 is required for infection of B cells (Wang *et al.*, 1998, Li *et al.*, 1995). Gp42 is poorly conserved with no predicted homologue in any

other human herpesvirus (Li *et al.*, 1995), however there is a homologue in ORF51 of EHV-2 (Telford *et al.*, 1995) and there may be a functionally similar protein in HCMV (Huber & Compton, 1997, Li *et al.*, 1997).

GM appears to have a role in penetration but is not essential for EBV replication (Lake *et al.*, 1998). GM is predicted to be a highly hydrophobic type 3 membrane protein with 8 putative transmembrane domains (Baer *et al.*, 1984) as are all studied herpesvirus gM homologues i.e. pseudorabies virus, HSV-1, HCMV, EHV1 (Dijkstra *et al.*,1996, Baines & Roizman, 1993, Lehner *et al.*, 1989, Osterreider *et al.*, 1996). Similar to the scenario for gH and gL, the presence of gM is required for authentic processing of gN a small (15kDa) type I membrane protein (Lake *et al.*, 1998). GN and gM can be detected in the plasma membrane on infected cells, although their presence has not been shown on the virion membrane they are likely to be present. The role of gM and GN has not been fully elucidated. The function of EBV gp150 (BDLF3) is unknown, a gp150 negative virus had no detectable replication defect *in vitro* (Borza & Hutt-Fletcher, 1998).

1.7.5 EBV gB

Unlike HSV-1 gB, EBV gB is not present on the viral surface and plays no role in attachment or entry (Gong & Kieff, 1990). However, gB is essential for productive infection of cells (Herrold *et al.*, 1996, Lee & Longnecker, 1997). This is reminiscent of HSV-1 gK, which is also an essential gene but is not a virion component (Hutchinson & Johnson, 1995). Gp110 is detained in the ER, has N-linked glycosylation of the pre-golgi, high mannose type and localises to the nuclear membrane (Gong & Kieff, 1990, Papworth *et al.*, 1997, Lee, 1999). GB of KSHV has been shown to have a similar localisation pattern to gB of EBV, demonstrating the close relationship of these viruses, however there is not sufficient functional homology to allow efficient complementation of an EBV mutant lacking gB with gB of KSHV (Pertel *et al.*, 1998). Localisation of gB homologues at the nuclear membrane, from where herpesviruses bud, suggests they may be involved in the egress of the virus from the nucleus.

1.7.6 Glycoproteins of MHV-68

From the genetic analysis of MHV-68 homologues for the genes encoding the four glycoproteins gB, gH, gL and gM have been identified (Virgin *et al.*, 1997). Of these only gB has been characterised for MHV-68. Investigation into the nature of the MHV-68 gB was considered important as it provides a good reflection on the similarity of MHV-68 with gammaherpesviruses such as EBV. Gp110 of EBV is unusual compared to the HSV gB prototype (Gong & Kieff., 1990). The gene encoding gB of MHV-68 is located to the left end of the genome (ORF 8) similar to the position of g110 in EBV (BALF4). MHV-68 gB has 42%, 49% and 26% codon identity with gB of EBV, HVS and HSV-1 respectively. As with gp110 and gB of KSHV, gB of MHV-68 has N-linked glycosylation of the high mannose type, is retained in the ER; localising to the nuclear membrane and is not detected in mature virions (Stewart *et al.*, 1994a).

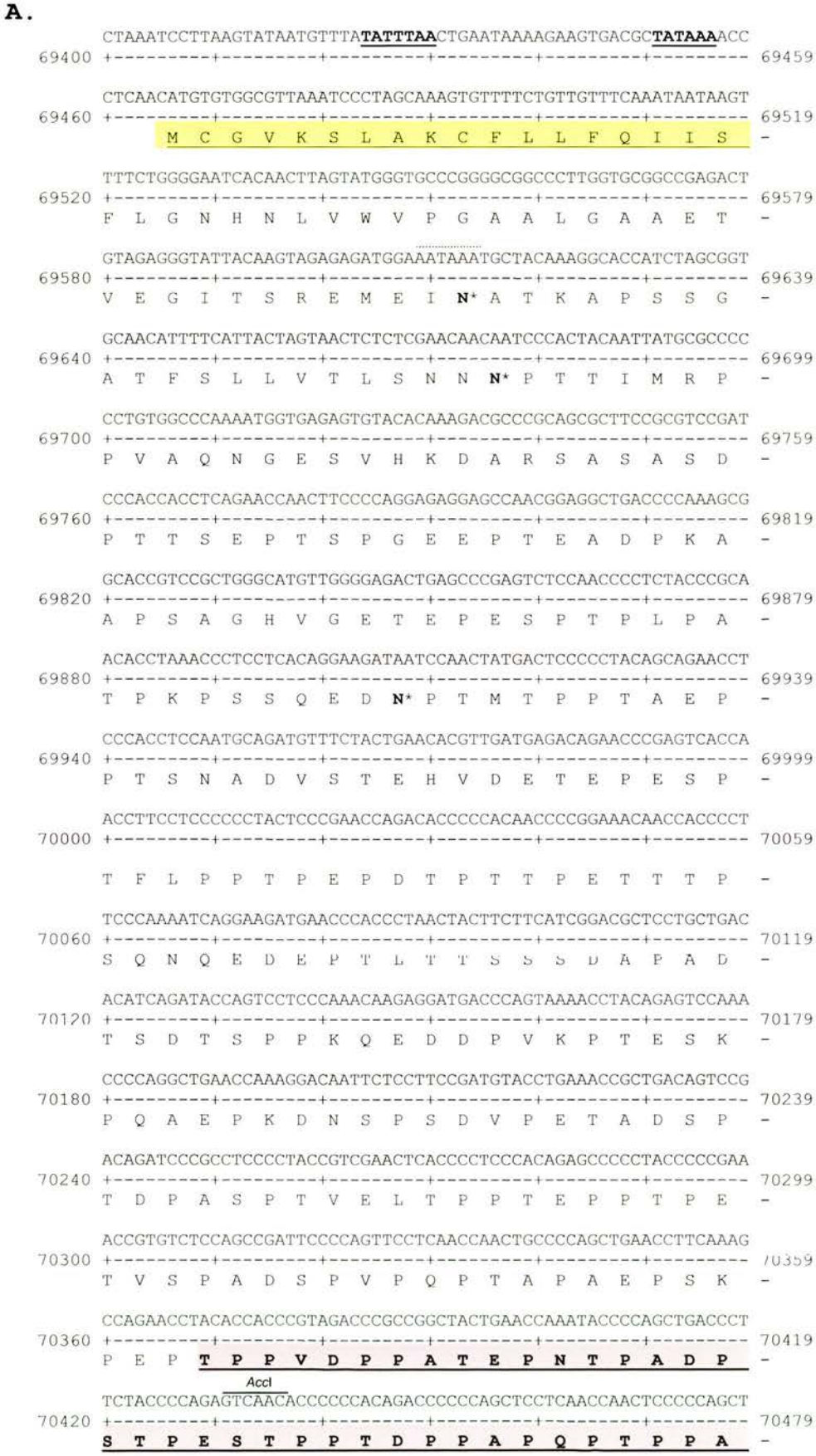
1.7.7 MHV-68 Glycoprotein 150 (gp150)

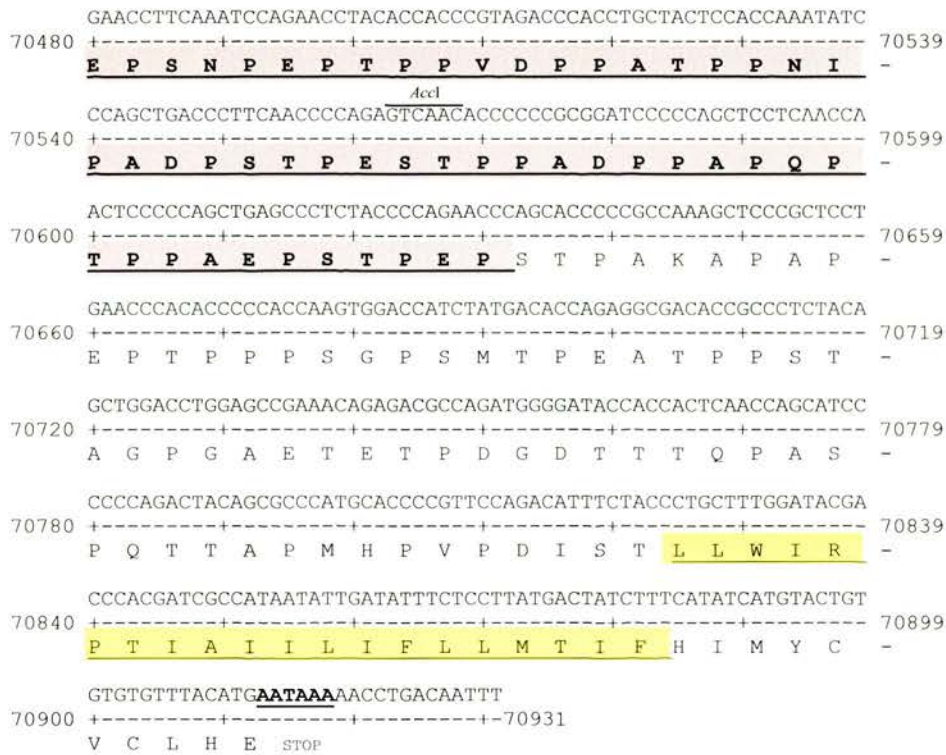
Glycoprotein 150 (gp150) is the only other MHV-68 glycoprotein to have been characterised, selected for study as a potential homologue of gp340/220 of EBV. Like gp340/220, the gene encoding gp150 is in a central genomic position. Comparison of FASTA[1] scores of positionally analogous glycoprotein amino acid sequences for other gammaherpesviruses, relative to 2,087 of MHV-68 showed only gp340/220 to have significant homology (FASTA score 278). Gp150 has 25% identity and 40% similarity at the amino acid level with gp340. The gene encoding gp150, M7, is not spliced and the predicted protein consists of 483 amino acids. Gp150 is a type one transmembrane glycoprotein with a long hydrophilic domain flanked by a hydrophobic signal peptidase cleavage site at the amino terminus and a hydrophobic transmembrane domain near the carboxy terminus (refer to figure 1.8). Gp150 has a high content of serine and threonine (24%) and there are three potential sites for N-linked glycosylation, only one of which appears to be used (deduced from enzymatic digestion analysis, plus two of the potential sites contain a proline). Gp150 has an unusually high proline content (23%) predominantly contained in a

[1] FASTA is an option of the UW GCG package which allows comparisons of protein sequences (Devereux *et al.*, 1984)

domain of proline rich repeats, 22 amino acids in length. This repeat region is similar to a domain of gp340/220, and is thought to effect the large apparent molecular weight of 150,000, observed for gp150 on SDS-PAGE gels (the predicted molecular weight of the core unglycosylated protein is 48,000). A precursor to gp150, with a molecular weight (MW) of 130,000 and high mannose N-linked glycans, is formed but only the mature 150,000 MW gp150 is expressed on the surface of infected cells and virion particles. An additional minor unglycosylated protein with a MW of 110,000 can be identified on blots probed for gp150, it is not a precursor of gp150 and is thought to arise from a downstream translational initiation site in the gp150 mRNA. Antibodies to gp150 have been reported to neutralise MHV-68 infection *in vitro* in the absence of complement (Stewart *et al.*, 1996). Similarly antibodies to Gp340/220 neutralise EBV infection *in vitro*. Gp340/220 determines the B cell specificity of EBV through binding CD21, predominantly a B cell marker and complement receptor (Nemerow *et al.*, 1989). It is thought that gp150 could have a similar function to gp340/220 i.e. in facilitating virus binding to cells, possibly determining the B cell specificity of MHV-68, however gp150 does not contain the CD21 binding peptide motif present in gp340/220.

A.


CTAAATCCTTAAGTATAATGTTTATTATTTAACTGAATAAAAGAAGTGACGCTATAAAACC
69400 69459
CTCAACATGTGTGGCGTTAAATCCCTAGCAAAGTGTTTTCTGTTGTTTCAAATAATAAGT
69460 69519
M C G V K S L A K C F L L F Q I I S -
TTTCTGGGGAATCACAACTTAGTATGGGTGCCCGGGGCGGCCCTTGGTGCGGCCGAGACT
69520 69579
F L G N H N L V W V P G A A L G A A E T -
GTAGAGGGTATTACAAGTAGAGAGATGGAAATAAATGCTACAAAGGCACCATCTAGCGGT
69580 69639
V E G I T S R E M E I N* A T K A P S S G -
GCAACATTTTCATTACTAGTAACTCTCTCGAACAACAATCCCACTACAATTATGCGCCCC
69640 69699
A T F S L L V T L S N N N* P T T I M R P -
CCTGTGGCCCAAAATGGTGAGAGTGTACACAAAGACGCCCGCAGCGCTTCCGCGTCCGAT
69700 69759
P V A Q N G E S V H K D A R S A S A S D -
CCCACCACCTCAGAACCAACTTCCCCAGGAGAGGAGCCAACGGAGGCTGACCCCAAAGCG
69760 69819
P T T S E P T S P G E E P T E A D P K A -
GCACCGTCCGCTGGGCATGTTGGGGAGACTGAGCCCGAGTCTCCAACCCCTCTACCCGCA
69820 69879
A P S A G H V G E T E P E S P T P L P A -
ACACCTAAACCCTCCTCACAGGAAGATAATCCAACTATGACTCCCCCTACAGCAGAACCT
69880 69939
T P K P S S Q E D N* P T M T P P T A E P -
CCCACCTCCAATGCAGATGTTTCTACTGAACACGTTGATGAGACAGAACCCGAGTCACCA
69940 69999
P T S N A D V S T E H V D E T E P E S P -
ACCTTCCTCCCCCCTACTCCCGAACCAGACACCCCCACAACCCCGGAAACAACCACCCCT
70000 70059
T F L P P T P E P D T P T T P E T T T P -
TCCCAAAATCAGGAAGATGAACCCACCCTAACTACTTCTTCATCGGACGCTCCTGCTGAC
70060 70119
S Q N Q E D E P T L T T S S S D A P A D -
ACATCAGATACCAGTCCTCCCAAACAAGAGGATGACCCAGTAAAACCTACAGAGTCCAAA
70120 70179
T S D T S P P K Q E D D P V K P T E S K -
CCCCAGGCTGAACCAAAGGACAATTCTCCTTCCGATGTACCTGAAACCGCTGACAGTCCG
70180 70239
P Q A E P K D N S P S D V P E T A D S P -
ACAGATCCCGCCTCCCCTACCGTCGAACTCACCCCTCCCACAGAGCCCCCTACCCCCGAA
70240 70299
T D P A S P T V E L T P P T E P P T P E -
ACCGTGTCTCCAGCCGATTCCCCAGTTCCTCAACCAACTGCCCCAGCTGAACCTTCAAAG
70300 70359
T V S P A D S P V P Q P T A P A E P S K -
CCAGAACCTACACCACCCGTAGACCCGCCGGCTACTGAACCAAATACCCCAGCTGACCCT
70360 70419
P E P T P P V D P P A T E P N T P A D P -
AccI
TCTACCCCAGAGTCAACACCCCCCACAGACCCCCCAGCTCCTCAACCAACTCCCCCAGCT
70420 70479
S T P E S T P P T D P P A P Q P T P P A -



B.

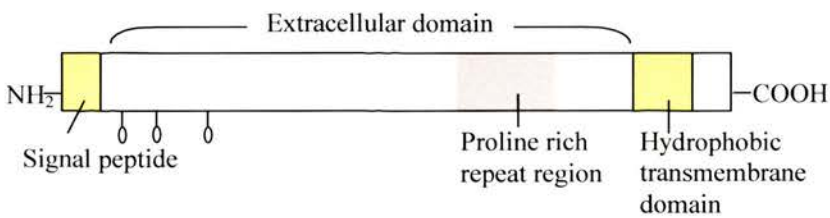


Figure 1.8. A. The sequence of the MHV-68 open reading frame 51 encoding gp150. The DNA sequence is numbered according to the complete MHV-68 genome numbering described by Virgin *et al.*, 1997. The end of the sequence represents the end of the cDNA obtained by RT-PCR (Stewart *et al.*, 1996). The predicted amino acid sequence is shown below the DNA sequence. The sites of consensus promoter and polyadenylation signal sequences upstream and downstream of the ORF (boldface and underlined), the extent of the two hydrophobic domains (highlighted in yellow), the sites of potential N-linked glycosylation (bold face and marked with an asterix), and the extent of a proline-rich 22-amino-acid repeat unit (highlighted in grey) are indicated (adapted from Stewart *et al.*, 1996). The location of the *Acc*I restriction enzyme sites are shown and the consensus polyadenylation signal for ORF50 is marked by the dotted line above the nucleic acid sequence. **B**. A schematic representation of the gp150 encoding gene, lollipop symbols represent potential sites of N-linked glycosylation.

1.7.8 KSHV K8.1

KSHV encodes K8.1 which has the same genetic locus and orientation as gp150 and gp340/220 of MHV-68 and EBV respectively (Raab *et al.*, 1998). K8.1 is spliced, encodes a glycoprotein of 228 amino acids (201 amino acids when mature after cleavage of the signal sequence), that resolves at the relatively small size of 37kDa. A transcript encoding a smaller 167 amino acid product designated K8.1.B, derived from ORF K8.1, has been identified that shares similar amino and carboxyl termini with ORF K8.1 but with an in-frame deletion (Chandran *et al.*, 1998). K8.1 is similar to gp150 being a type I transmembrane glycoprotein with 4 potential N linked glycosylation sites and a high content of serine and threonine residues (21%). Like gp150 and gp340/220, K8.1 localises to the surface of virions and infected cells (Chandran *et al.*, 1998, Li *et al.*, 1999). Antibodies to K8.1 are detected regularly in individuals with KS and have the potential to be used as contributory serological markers (e.g. together with anti-latency-associated nuclear antigen (LANA) antibodies) for infection by KSHV (Li *et al.*, 1999, Zhu *et al.*, 1999). Studies into the function of K8.1 and whether antibodies against K8.1 are neutralising have not yet been published however it is very possible that K8.1 shares a similar role to gp150.

As yet, other than K8.1, limited attention has been directed to the glycoproteins of KSHV which include the conserved herpesvirus glycoproteins. Like gp110 of EBV, gB encoded by KSHV has been shown to lack Golgi processing and localise to the endoplasmic reticulum and nuclear membrane. Although gB of KSHV can interact with EBV gp110 there is not sufficient functional homology to enable efficient complementation of gp110 negative EBV mutants (Pertel *et al.*, 1998).

1.7.9 Immune Response to Glycoproteins

For most enveloped viruses antibodies against one or more of the membrane glycoproteins neutralises infectivity. Neutralisation of infection can occur by physically inhibiting virus attachment to cells or at a further entry step of infection (i.e. post attachment) depending on the function and location of the recognised glycoprotein. Glycoprotein specific antibodies can reduce virus infectivity by

aggregating virus particles and target virions for phagocytosis and complement induced destruction. In addition these antibodies can identify productively infected cells, expressing viral glycoproteins, for destruction by antibody dependent cellular cytotoxicity and complement lysis. The significant response and protective effects of antibodies against viral glycoproteins have been demonstrated for a wide range of viruses e.g. antibodies to the envelope glycoprotein of rabies (Rhabdovirus) blocked infection *in vitro* and *in vivo* prevented rats from undergoing lethal disease, (Dietzschold *et al.*, 1992). Monoclonal antibodies to the H glycoprotein of measles exerted a therapeutic effect in virus infected cells in culture (Schneider-Schaulies *et al.*, 1992). Antibodies against glycoproteins of respiratory syncytial virus (RSV) administered parenterally or direct to the lungs in cotton rats had a therapeutic effect against RSV infection (Crowe *et al.*, 1994, Crowe *et al.*, 1998). Antibodies against HA and NA are associated with resistance to influenza infection (Clements *et al.*, 1986, Couch & Kasel, 1983). Essentially all of the neutralising antibody activity to HIV-1 in infected hosts is directed against the envelope glycoproteins gp120 and gp41. B and T cell responses are directed to the V3 loop of gp120 (Parker *et al.*, 1988, Robert-Guroff *et al.*, 1994, Takahashi *et al.*, 1988, Carrow *et al.*, 1991, Emini *et al.*, 1992, Javaherian *et al.*, 1989). Single amino acid substitutions in gp120 within the V3 region have been identified which confer the resistance of immune escape mutants of HIV-1 to neutralising antiserum (Watkins *et al.*, 1993). The glycoproteins of HSV are highly immunogenic, gD and gB are particularly effective at inducing neutralising antibodies (Blacklaws *et al.*, 1990). In animal models the levels of neutralising antibodies against glycoproteins gB and gD of HSV have been shown to correlate with the degree of protection (Meignier *et al.*, 1987, Stanberry *et al.*, 1987).

Although much emphasis is placed on the ability of glycoproteins to generate neutralising antibodies glycoproteins are also recognised by T cells and glycoprotein specific CTLs can effect protection against virus infections. For example, in experimental guinea pigs protection from Ebola virus infection has been achieved by immunisation with plasmids encoding GP and SGP prior to challenge (Xu *et al.*, 1998). The antibody response was weak and non-neutralising and it appears that

cytotoxic T cells to epitopes of GP played an important role in control of virus infection.

1.8.1 Viral Vaccines Based on Glycoproteins

Among the varying strategies for vaccination, glycoproteins have received much attention as potential vaccine immunogens for a wide range of viruses. Glycoprotein based vaccines can take many forms including – purified native protein, recombinant proteins expressed independently of other viral components, protein subunit vaccines, glycoproteins combined into immune stimulating complexes (ISCOMs) and liposomes, peptide epitope vaccines, recombinant viral vectors (incorporation of the glycoprotein encoding gene into viral vector such as vaccinia virus) and DNA vaccines (the glycoprotein encoding gene is introduced directly into an individual to be expressed by recipients own cells). The different vaccine forms have their inherent advantages and disadvantages. Currently there is no licensed vaccine available for human use based on a glycoprotein however, viral glycoproteins are the focus of much research and have the potential to emerge in the future.

For HSV gD and gB have been manipulated and tested in a number of vaccine forms e.g. a gD vaccinia recombinant (Straus *et al.*, 1994, Straus *et al.*, 1993), GD and GB have been formed into a protein subunit vaccine (Straus *et al.*, 1997) and a gD DNA vaccine has been developed (Kriesel *et al.*, 1996). The success of these have been limited and is thought to reflect their restricted immunogenicity. GB of CMV is the major surface antigen and important target of neutralising antibodies (Britt *et al.*, 1988) and cellular immune responses. A subunit vaccine consisting of gB coupled with a lipid emulsion adjuvant induced high titres of CMV specific neutralising antibodies and is a serious candidate for human clinical trials (Pass *et al.*, 1995). Expression of CMV gB in canarypox was achieved in order to improve CTL responses to gB (Gonczol *et al.*, 1995). DNA vaccination of glycoprotein genes of pseudorabies virus have been tested for protection against pseudorabiesvirus infection in pigs (Haagmans *et al.*, 1999). Table 1.4 provides a very limited number of examples of different experimental viral vaccines that have been developed based on glycoproteins. The vaccine forms generated and potential of gp340/220 as an

immunogen to provide protection against EBV infection are discussed in sections 1.8.7-1.8.9.

As well as glycoproteins being the direct object of a vaccination strategy – generation of attenuated viruses for vaccination has been approached by making viruses deficient in a glycoprotein known to be important for virus replication and virulence – for example a gH HSV deletion mutant (Boursnell *et al.*, 1997).

When selected for use as an immunogen the amount of variation of a glycoprotein between isolates of a virus must be appreciated. Fortunately, herpesvirus glycoproteins are very stable between isolates in comparison to glycoproteins such as gp120 of HIV and haemagglutinin of influenza. The antigenic variation of the influenza HA is the basis of the emergence of 'flu epidemics and pandemics.

Virus	Immunogen	Vaccine Form	Reference
HIV	Gp160	DNA vaccine	Kaneko *et al.*, 2000
HSV	gB & gD	Protein subunit	Straus *et al.*, 1997
HSV	gD	DNA vaccine	Manickan *et al.*, 1995
Pseudorabies virus	gD	DNA vaccine	Haagmans *et al.*, 1999
EBV	Gp340/220	Vaccinia recombinant	Mackett *et al.*, 1996
EHV-1	gC	DNA vaccine & Vaccinia recombinant	Huemer *et al.*, 2000
CMV	gB	Protein subunit	Pass *et al.*, 1995 & 1999
CMV	gB	DNA vaccine	Endresz *et al.*, 1999
Rabies	Glycoprotein	Canary pox recombinant	Taylor *et al.*, 1991
Influenza	Haemagglutinin & Neuraminidase	Liposome/protein complex	Babai *et al.*, 1999
RSV	Glycoprotein G	Synthetic peptide	Trudel *et al.*, 1991

Table 1.4. Examples of experimental viral vaccines based on glycoproteins.

1.8.2 EBV Vaccine Development

When considering vaccine development the beneficial potential to human health has to be evaluated. With this in mind, there are currently three main areas where a vaccine against EBV could make a significant impact these include the prevention of infectious mononucleosis, eBL and NPC. In western societies the focus is on combating IM, although perhaps less worthy, this is likely to be the most commercially attractive target being a problem of more financially buoyant countries. IM has been reported to be the second most frequent disease in

adolescents in the US (Wedderburn *et al.*, 1984), and although death is a rare outcome, IM can be fatal (Bailey, 1994). The prevention of endemic Burkitt's lymphoma in Africa and Papua New Guinea has long been goal however the lack of profitability has impeded progress. Populations with a high risk of NPC would greatly benefit from an effective vaccine against NPC, small human trials testing an EBV vaccine have been done in China (Gu *et al.*, 1995).

1.8.3 Potential EBV Vaccine Beneficiaries

EBV naïve individuals

The question of who would benefit from receiving a vaccine against EBV must take into consideration that currently 95% of the world human population is EBV positive. Young children, naïve to EBV are obvious candidates to be vaccinated against EBV in an effort to prevent primary infection and so avoid IM, NPC and eBL. Lifelong protection may not be achieved but a delay in EBV infection would possibly limit the potential development of NPC by enabling the individual to contain reactivating virus more efficiently. To prevent eBL ideally a vaccine would prevent establishment of latency in lymphocytes. If total protection is not possible, reducing the pool of latently infected B cells may reduce the incidence of BL. Reduction of lytic replication during primary EBV infection may subsequently result in less latently infected B cells. Infectious mononucleosis may be avoided or delayed in individuals primed against EBV able to limit the proliferation of virus carrying B cells. In summary, if a vaccine that is completely effective at preventing EBV infection is unlikely the next best scenario is a vaccine that would reduce the pool of latently-infected B cells, the ability of EBV to reactivate and so the development of EBV clinical disorders.

EBV infected individuals

Vaccinating individuals that are already infected with EBV offers potential benefits, this approach may positively modify the immune response to EBV limiting the development of late complications of EBV infection. In NPC a marker prior to disease development is the presence of serum IgA antibodies to lytic antigens of

EBV (Zeng *et al.*, 1985). It has been suggested that excessive replication of EBV may be part of the process of NPC occurrence. A vaccine that modified the individuals immune response in a way that would subdue EBV lytic replication could possibly prevent NPC from developing. In China NPC commonly occurs in adults of about 40 years of age and may be due to a waning immunity to EBV. A vaccine that boosted the immunity of an individual already infected with EBV to lytic EBV antigens may help to maintain life long immunity against EBV reactivation. An alternative vaccine that targets EBV latent antigens could stimulate cell mediated immunity to eliminate infected B cells post primary infection and reduce the pool of potential reactivatable and eBL cells.

Individuals with EBV associated disease

Therapeutic vaccination is another possibility i.e. an EBV vaccine administered to individuals actually presenting with EBV positive neoplasms would aim to specifically target the immune response to the tumours via EBV antigens expressed in the malignant cells e.g. EBNAs, LMP1 and LMP2a/2b and possibly also genes such as BZLF-1 (Cochet *et al.*, 1993). Regression of EBV induced human B cell lymphoproliferation and protection against LPD has been observed in SCID mice following the introduction of CTLs primed against EBV antigens (Buchsbaum *et al.*, 1996, Johannessen *et al.*, 1999). This work was directed at the development of T cell therapy to treat lymphoproliferative disorders primarily in the immunosuppressed transplant patient however it has implications for vaccine development suggesting if the CTLs present in immune intact individuals could be focused towards EBV antigens the EBV induced proliferation could be eliminated.

1.8.4 Immune Response to EBV

Primary infection of EBV induces a humoral immune response of antibodies specific to an array of EBV encoded proteins. During acute IM the initial antibody response consisting of IgM is directed specifically against lytic viral proteins including early antigens, viral capsid antigens and viral membrane antigens. Antibodies to EBNA 1 and EBNA 2 develop later. In acute IM the humoral response is considered ineffective as the antibody response to gp340, the most potent source of neutralising

antibodies, is lacking (Khanna *et al.*, 1995a). Gp85 the gH homologue of EBV is the focus of the antimembrane antigen antibody response rather than gp340 (Henle & Henle, 1979). The majority of the neutralising antibody response to EBV is directed to gp340/220 (Thorley-Lawson & Poodry, 1982). The antibody directed to EBNA and viral capsid antigens is thought to have no or little role in protection although remaining for life (Khanna *et al.*, 1995a).

After acute infection the IgM levels to EBV subside and EBV specific IgG is produced. Complement dependent neutralising antibody occurs early in acute IM and is followed by the development of complement independent neutralising antibody (Sairenji *et al.*, 1984). EBV production can be controlled by EBV antibodies, antisera against membrane antigens from IM patients correlates with inhibition of EBV replication *in vitro* (Sairenji *et al.*, 1991). Monoclonal antibodies against different epitopes of EBV gp340 have been shown to neutralise EBV infection and inhibit EBV release from infected cells (Thorley-Lawson & Geilinger, 1980).

Cell mediated immunity plays a major role in the control of primary and persistent EBV infection (Rickinson & Moss, 1997). During primary infection approximately 90% of the EBV specific CTLs are directed against lytic antigens (Tan *et al.*, 1999). The levels of latently infected B cells are regulated by cell mediated immune recognition of EBV immediate early and latent antigens expressed (Bogedain *et al.*, 1995 & Khanna *et al.*, 1995b). The importance of this cell mediated control is clearly demonstrated when immunosuppression of CTLs in transplant patients results in EBV induced lymphoproliferative disease. EBV specific CTLs that have been isolated from patients with post transplant lymphoproliferative disease, expanded *in vitro* and infused back into the sufferer have successfully suppressed the development of PTLD (Rooney *et al.*, 1995). CTLs can inhibit EBV induced B cell transformation following recognition of MHC I processed viral antigens on the surface of infected B cells (Bejarano *et al.*, 1988 & 1990). In EBV seropositive individuals EBV specific CTLs can readily be isolated which are thought to persist for life suppressing the potential development of EBV associated disorders by constant immune surveillance (Moss *et al.*, 1979, Rickinson *et al.*, 1979).

Most of the information on the cell mediated immune response to EBV surrounds the CTLs directed against EBV latent antigens there is a deficit of information of those to the lytic cycle antigens due to the lack of a cell line permissive for lytic EBV replication. $CD4^+$ T cells specific for gp340 have been identified (Wallace *et al.*, 1991). It is possible that $CD4^+$ T cells activated by viral antigens are involved in the control of infection by modulating a local inflammatory response through cytokine release. *In vitro* lytically infected cells have been shown to be more sensitive to NK induced cell lysis (Blazar *et al.*, 1980). Also, cells expressing gp340 at their surface are targets of EBV specific antibody dependent cellular cytotoxicity (Khyatti *et al.*, 1991).

1.8.5 Desired Immune Response Generated by a Vaccine to EBV

The two specific facets of immunity to EBV are the humoral and CTL response. In designing a vaccine the type and specificity of immunity against the virus desired must be identified. In view of the oral route of EBV transmission a vaccine that produced protective immunity at the mucosal surfaces may be especially beneficial aiming to prevent initial EBV infection. The humoral immune response to EBV could be modified to prevent initial infection of cells by antibody neutralisation of virus blocking attachment and entry of the virus. The cytotoxic T cell response could be primed to more effectively clear infected cells.

1.8.6 EBV Vaccine Development

With regard to EBV vaccine development, two main directions have been taken. One area has targeted the immunogenicity of the lytic antigen gp340/220 concentrating principally on the ability of this membrane glycoprotein to induce neutralising antibodies. Strategies based on gp340 in general aim to limit lytic replication, preferably for the prevention of primary infection of EBV. The other focus of EBV vaccine design has been on a vaccine that generates an efficient EBV specific CTL response targeting the destruction of cells infected with EBV as opposed to preventing initial infection of cells. Latently expressed EBV antigens have been subject to most investigation in this area in an attempt to generate CTLs in the individual that will recognise and eliminate latently infected B cells in the host.

1.8.7 Gp340/220 Based Vaccines

Due to the potential oncogenic nature of EBV an attenuated virus vaccine is not favoured therefore alternative strategies have been considered. As gp340 includes the major neutralising determinants for EBV it is an obvious candidate for exploitation as a vaccine immunogen and importantly gp340/220 is highly conserved between EBV types 1 and 2 (Lees *et al.*, 1993). In this vein many different methods have been employed to express and generate purified and viral vector incorporated gp340 including purification from the membranes of the EBV infected LCL B95.8 (Morgan *et al.*, 1984a & 1988a, Randle *et al.*, 1985, Epstein *et al.*, 1985) expression from mammalian expression vectors in different mammalian cell types (Conway *et al.*, 1989, Madej *et al.*, 1992, Motz *et al.*, 1986 & 1987, Whang *et al.*, 1987), baculovirus (Zhang *et al.*, 1993, Nuebling *et al.*, 1992) and yeast expression systems (Emini *et al.*, 1988). The different expression methods have their inherent pros and cons. Expression of fragments of gp340 in bacteria produced peptides that were unable to induce neutralising antibodies (Pither *et al.*, 1992, Zhang *et al.*, 1991) The purification of gp340 from infected cells was inefficient, resulting in very small quantities of protein which would not ultimately be suitable for human use due to the use of phorbol esters and sodium butyrate to stimulate productive replication of EBV. Similarly, a bovine papilloma expression system used to generate gp340 may encounter resistance for human use the vector being based in a virus with oncogenic potential. Activity continues in analysing the production and potential of gp340 protein based vaccines (Jackman *et al.*, 1999). Recombinant viruses such as adenovirus 5 and vaccinia virus engineered to express gp340 have also been generated (Ragot *et al.*, 1993, Mackett *et al.*, 1985). In general the forms of gp340 produced in recombinant expression systems in mammalian cells have proved to be the most desirable being indistinguishable by monoclonal antibodies from the wild type form of gp340 (Whang *et al.*, 1987, Madej *et al.*, 1992).

1.8.8 Gp340 Based Vaccination Trials

A number of vaccination trials to assess protection against EBV induced disease have been done with gp340 based vaccines in several animal models. Promisingly a gp340 vaccinia recombinant (strain WR) was successful at protecting cotton top tamarins (*Saguinus oedipus oedipus*) from a lethal lymphomagenic dose of EBV. Likewise a gp340 expressing adenovirus 5 recombinant induced protective immunity against EBV induced lymphomas in cotton top tamarins. However, the mechanism of protection is not fully understood as gp340 specific antibody levels did not correlate with protection in these studies (Morgan *et al.*, 1988b, Ragot *et al.*, 1993). Decreased EBV replication was observed in common marmosets immunised with the same vaccinia virus expressing EBV gp340 (Mackett *et al.*, 1996). Reduced viral shedding was achieved following EBV challenge of common marmosets that had been immunised with purified gp340 derived from a bovine papilloma virus expression system (Cox *et al.*, 1998). These studies show promise however the mechanism of protection has not been clearly defined and does not appear to be due to neutralising antibodies against gp340, cell mediated immunity has been implicated in providing the protection.

Over the past years a vaccine based on gp340, or a surface glycoprotein of any herpesvirus for that matter, has aimed to induce immunity to prevent preliminary infection usually by the induction of neutralising antibodies. The potential of gp340 as a useful CTL target is gaining more attention. Gp340 has been shown to prime cytotoxic T cells also (Wilson *et al.*, 1999). T cells that prevent EBV transformation *in vitro* have been generated by gp340 iscom stimulation (Bejarano *et al.*, 1990). Plus, prior immunization of HLA A2/Kb transgenic mice with gp340 and gp85 CTL epitopes induced a strong epitope-specific CTL response and afforded protection against gp85- or gp340-expressing vaccinia virus challenge (Khanna *et al.*, 1999). In addition, T helper cells specific for gp340 have been detected in EBV seropositive individuals (Ulaeto *et al.*, 1988, Wallace *et al.*, 1991).

1.8.9 Human Trials

Human trials using a recombinant vaccinia virus (Chinese strain Tien Tan) expressing gp340 have been done in China (Gu *et al.*,1991 & 1995). The gp340 vaccinia recombinant was initially tested for toxicity in mice and rabbits and then administered to adults. The individuals had a weak or undetectable response with no change in neutralising antibodies. Subsequently the vaccine was administered to children of about 10 years old who did show a clear specific antibody response. The lack of response in the adults may have been due to previous exposure to vaccinia virus. As very young children are likely to be the ultimate target of a vaccine to prevent infection being naïve to EBV a trial involving 19 infants aged 9-12months was performed. Prior to the trial all the participants tested seronegative for EBV and vaccinia virus. The gp340 vaccinia virus recombinant did appear to provide protection to 6/9 children who remained EBV negative a year post vaccination unlike the 10 control individuals, whether this is long term immunity is not clear. Three years post vaccination 5/9 were EBV negative, those that had been infected with EBV had lower sera titres than infected controls suggesting lower EBV lytic replication and therefore partial protection against EBV. These results are promising however they must be viewed with caution due to the limited numbers involved in the trials.

1.9.1 EBV T Cell Epitope Based Vaccines

An alternative approach to the development of a vaccine strategy against EBV is that based on CTL epitopes. The aim is to immunise the $CD8^+$ T cell repertoire using formulations of synthetic peptides which mimic immunodominant epitopes known to be recognised by the EBV induced CTL response. This approach to vaccination has mainly aimed to target latently infected B lymphocytes rather than virions and lytically infected cells at the primary site of infection. This would in theory reduce virus-induced B cell proliferation but not prevent initial infection of cells and therefore latency could be established albeit in a reduced number of B lymphocytes.

For EBV, the CTL epitopes focused upon for the incorporation into a vaccine are those of latency associated proteins. The low levels of EBV infected cells in healthy

EBV carriers are thought to be regulated in the main by CTLs for EBNA 3 and LMP. EBNA3 has particularly immunodominant epitopes that are commonly recognised by EBV specific CTLs in healthy EBV carriers (Murray *et al.*, 1992, Burrows *et al.*, 1994). The tumour cells of PTLD express all of the EBV latent genes of which EBNA 3 proteins are the main CTL targets (Khanna *et al.*, 1995a).

As different MHC class I alleles present different peptides the diversity of HLA MHC class I restriction within the human population increases the difficulty of developing a peptide vaccine. A peptide vaccine that is effective for the majority of the human population is likely to need a variety of epitopes incorporated. There are reports of investigations into the EBV epitopes recognised by $CD8^+$ T cells in particular MHC backgrounds. There is also evidence that recombinant vectors expressing the epitopes in a chain is feasible i.e. the epitopes are processed and presented effectively able to reactive memory specific CTLs *in vitro* (Thomson *et al.*, 1995). Reports also suggest that allelic variation in the repertoires of T-cell receptors could limit the CTL reactivity to a particular epitope, another obstacle for peptide vaccinology (Schmidt *et al.*, 1991). Analysis of the conservation of any chosen epitope among strains of EBV must also be done. Vaccines based on latent antigen CTL epitopes have been designed (Khanna *et al.*, 1995a). Reduction in the numbers of latently infected cells may be sufficient to reduce the incidence of EBV associated diseases however sterile immunity would be preferable.

1.9.2 AIMS

The aim of this project was to characterise the function of the virion membrane glycoprotein gp150 with respect to MHV-68 replication. Approaches taken were to make a MHV-68 recombinant virus unable to express gp150 and to assay the potential binding function of gp150 in isolation as a purified protein. In addition, the potential of gp150, administered in the form of a DNA vaccine in conjunction with a gp150 expressing recombinant vaccinia virus, to protect inbred mice against MHV-68 challenge was subject to a preliminary investigation.

2. MATERIALS & METHODS

Unless otherwise stated all standard laboratory reagents were purchased from Sigma or BDH- Merck.

Unless otherwise stated reagents were made up in water (H_2O) prepared using a 18 Ω Millipore water filter unit.

MOLECULAR BIOLOGY

2.1.1 Plasmids

Plasmid	Size	Description	Selection	
			E. coli	Mam
pSP72/gp150	4 kb	Vector pSP72 (Promega) with the gene for gp150 inserted between restriction sites *Hind*III and *Eco*RI (Stewart *et al.*, 1996). Supplied by Dr. J P Stewart.	Amp	NA
pBluescript II KS	2.96kb	Cloning vector (Promega)	Amp	
pBabe/puro	5.1 kb	Mammalian expression vector based on Moloney murine leukemia virus (Morgenstern & Land 1990)	Amp	Puro
pIND/Hygro	5.2kb	Ecdysone inducible mammalian expression vector (Invitrogen).	Amp	Hygro
pIND/Hygro/*LacZ*	8.2kb	Positive control vector, encoding the β-galactosidase gene, for mammalian transfection and expression in the ecdysone inducible expression system (Invitrogen).	Amp	Hygro
pIND(SP1)/Hygro	5.2kb	A version of pIND/Hygro with three binding sites for the transcription factor SP1 to increase expression levels five fold over those obtained with pIND/Hygro. (Invitrogen)	Amp	Hygro
pIND(SP1)/Hygro /*LacZ*	8.2kb	Positive control vector, encoding the β-galactosidase gene, for mammalian transfection and expression in the ecdysone inducible expression system (Invitrogen).	Amp	Hygro
pVgRXR	8.7kb	Invitrogen vector encodes the heterodimeric ecdysone receptor, under the constitutive CMVIE promoter, for the ecdysone inducible mammalian expression system.	Zeocin	Zeocin
pEGFP-C1	4.7 kb	Clontech plasmid encoding green fluorescent protein (GFP) gene cassette gfp-C1; the gfp gene with a CMV immediate early promoter and a SV40 polyadenylation signal.	Kan	Neo
pGEX-2T	4.9kb	Vector for the inducible prokarytic expression of recombinant proteins fused to glutathione-S-transferase derived from *Schistosoma japonicum*.	Amp	NA
pET-22b(+)	5.5kb	Vector for the inducible prokaryotic expression of recombinant hexahistidine tagged proteins under the control of the T7 RNA polymerase promoter.	Carb/ Amp	NA
pMV10	4.7kb	Cloning vector, encodes β-galactosidase under the constitutive CMVIE promoter. Supplied by Dr. J.P. Stewart	Amp	
pcDNA3	5.4kb	Invitrogen mammalian expression vector containing CMVIE promoter for constitutive expression of cloned gene.	Amp	Neo
pcDNA3.1(+)	5.4kb	Invitrogen mammalian expression derived from pcDNA3.	Amp	Neo
pVR1255/gp150	6.4kb	pVR1255 (Hartikka *et al.*, 1996) with the gp150 encoding gene inserted between *Sal*I and *Bam*HI sites replacing the luciferase reporter gene. Supplied by Dr. F. Belardelli.	Kan	
pVR1255/IFN-αI	5.5kb	pVR1255 (Hartikka *et al.*, 1996) with the interferon alpha I (IFN-αI) encoding gene inserted between *Not*I and *Bam*HI sites replacing the luciferase reporter gene. Supplied by Dr. F. Belardelli.	Kan	
pAC8	4.5kb	Vector pCR2.1-TOPO (Invitrogen) containing the green fluorescent protein (GFP) expression cassette with the *gfp* MCS deleted between *Bgl*II and *Bam*HI. Supplied by Dr. D Roy.	Amp/ Kan	

Table 2.1. Cloning and expression plasmid vectors. Drug selection used in *E.coli* and in mammalian cells (Mam) – Amp– ampicillin, Carb- carbenicillin, Kan- kanamycin, Hygro- hygromycin, Neo- neomycin, Puro- puromycin, Zeo- zeocin. NA – not applicable. CMVIE – cytomegalovirus immediate early promoter.

2.1.2 Computer Assisted Analysis of DNA

For analysis of DNA sequences with regard to restriction mapping, sequence homology and protein translation the University of Wisconsin Genetics Computer Group (UWGCG) suite of programmes was accessed (Devereux *et al.*, 1984).

2.1.3 Quantification of DNA

DNA concentration was quantified by fluorometry using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc.) following the manufacturers instructions. Commercial DNA (Hoefer Pharmacia Biotech Inc.) of known concentration was used to calibrate the machine. DNA, 2µl, was added to 2ml of solution A (10µl Hoechst 33258 stock solution 1mg/ml and 10ml 10x TNE (100mM Tris, 10 mM EDTA $Na_2.2H_2O$, 2M NaCl, pH7.4) in 90 ml H_2O in a glass cuvette for quantification.

2.1.4 Polymerase Chain Reaction (PCR)

Hot start PCR reactions were performed using either *Taq* DNA polymerase (Boehringer Mannheim) or *Pfu* DNA polymerase (Stratagene). Reactions using *Taq* DNA polymerase were performed in a final volume of 100µl made up with H_2O containing 1µl dNTP mix (25mM for each nucleotide – dATP, dGTP, dCTP and dTTP), 10µl reaction buffer (Boehringer Mannheim), 1.5mM $MgCl_2$ (Boehringer Mannheim), 1µl of each of a sense and antisense primer (20 µM solution), 1µl target DNA (amounts varied from 1ng to 100ng) and 1µl *Taq* DNA polymerase (1unit/1µl). Reactions with *Pfu* DNA polymerase contained 10µl of *Pfu* buffer (Stratagene), 1µl dNTP mix (25mM for each nucleotide as above), 1 µl of each of a sense and antisense primer, 1µl *Pfu* DNA polymerase (1unit/µl) and 1µl of target DNA (amounts varied from 1ng to 100ng) made up to a final volume of 100µl with H_2O.

An Omnigene PCR machine (Hybaid) was used for the temperature cycles. The standard PCR cycling programme consisted of an initial stage of 95°C for 5 minutes and then the calculated primer annealing temperature for 1 minute at which point the temperature was held for the addition of the DNA polymerase. The temperature

programme continued with a sequence consisting of primer extension at 72°C, for the calculated duration required for the predicted product length, 95°C for 30 seconds for DNA denaturation and the primer annealing temperature for 1 minute. This sequence represented one cycle, routinely cycles were repeated 25 times followed by a final stage of extension at 72°C for 5 minutes. The resulting products of all PCR reactions were checked by agarose gel electrophoresis as described in section 2.2.5. For nested PCR 10µl of the product from the first round of PCR was used as the target for the second round of PCR.

2.1.5 RT-PCR

Following extraction of mRNA from cells in six well plates using the Boehringer Mannheim mRNA Capture Kit, as described in section 2.3.5., reverse transcription (RT) was performed in one of the two 0.2ml tubes used to capture the mRNA for each sample. To the 0.2ml tube, 50µl of reverse transcription mix consisting of 50mM Tris-HCl, 40mM KCl, 6mM MgCl, 10mM dithioerythrithol, pH8.3, 20nM of each of dATP, dCTP, dGTP and dTTP and 40 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Boehringer Mannheim) was added. Tubes were incubated at 37°C for 60 minutes on an Omnigene PCR machine (Hybaid). To remove the reverse transcription mix tubes were washed with the wash buffer provided as part of the mRNA capture kit. A 50µl PCR mix containing 0.5µl dNTP mix (25mM for each nucleotide – dATP, dGTP, dCTP and dTTP), 5µl reaction buffer (Boehringer Mannheim Ltd), 1.5mM $MgCl_2$ (Boehringer Mannheim Ltd), 0.5µl of each of a sense and antisense primer (20 µM solution) and 1µl *Taq* DNA polymerase (1unit/1µl) made up in H_2O, was then added to the tubes. The temperature cycles, as described in section 2.1.4 were performed using an Omnigene PCR machine (Hybaid). As a control for intact RNA and efficient reverse transcription primers for murine β-actin (intron spanning) shown in section 2.1.8 were used. To ensure the product detected was from cDNA and not from contaminating DNA one half of the samples was subjected to PCR without prior reverse transcription.

2.1.6 Calculation of Primer Annealing Temperature

For PCR with *Taq* DNA polymerase the annealing temperature for primers was calculated using the following formula:

$4 \times (N_A + N_C) + 2 \times (N_A + N_T) - 5 =$ Primer annealing temperature (^{o}C)

N equals the numbers of G, C, A and T nucleic acid bases in the primer complementary to the target in the first round of PCR. When using *Pfu* DNA polymerase the annealing temperature was reduced by 10°C from that calculated by the formula above. If a PCR failed to yield a product the PCR was repeated with annealing temperatures reduced by one or two degrees, the annealing temperatures displayed for primer pairs are those which successfully yielded a product.

2.1.7 Calculation of PCR Extension Time

Extension times were calculated on the basis of 1 kb/minute when using *Taq* DNA polymerase. In cases using *Pfu* DNA polymerase extension times based on 2kb/minute were used.

2.1.8 Primers

Oligonucleotides were purchased from MWG biotech. For primers in which not all of the sequence is complementary to the target sequence the bases complementary during the first cycle of PCR are underlined with a dashed line.

L50 – Left of gp150 (sense)
*Xho*I
5'-CGCGCTCGAGCTGACAGTGACACATGTTCC-3'
5' Location on MHV-68 genome: 69,236

L51 – In gp150 (antisense)
*Bgl*II *Acc*I *Eco*RI
5'-AGATCTGTCGACGAATTCTGCGGGCGTCTTTGTGT-3'
Location on MHV-68 genome: 69,743

L50 & L51 product size: 533bp
Target: MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temperature: 59°C, extension time: 45 seconds.

A52 - In gp150(sense)
*Eco*RI *Acc*I *Bgl*II
5'-GAATTCGTCGACAGATCTTTATTCATGATCATGTCTGTG-3'
5' location on MHV-68 genome: 70,956

B52 – Right of gp150 (antisense)
*Hin*dIII
5'-CGCGAAGCTTATTCTCCCTGAGCGATGGC-3'
5' location on MHV-68 genome: 71,380

A52 & B52 product size: 449bp
Target: MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temperature: 53°C, extension time: 45 seconds.

L50 & B52 product size: 965bp
Target: PCR products of A52 & B52 plus L50 & L51
PCR conditions with Taq DNA polymerase: annealing temperature: 59°C, extension time: 1 minute and 15 seconds.

IE1gp150 (sense)
*Hin*dIII
5'-GCGCAAGCTTCGCCGCCACCATGTGTGGCGTTAAATCC-3'
Feature (denoted by dotted line): Kozak translational initiation sequence
5' location on MHV-68 genome: 69,466

IE2gp150 (antisense)
*Xho*I
5'-CGCGCTCGAGTTATTCATGTAAACACACACAG-3'
5' location on MHV-68 genome: 70,917

IE1gp150 & IE2gp150 product: 1481bp
Target: MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temperature: 51°C, extension time: 2 minutes.

IA3 (sense)
*Hin*dIII
5'-CGCGAAGCTTAAAACCCTCAACATGTGTGG-3'
5' location on MHV-68 genome: 69,453

IA4 (antisense)
*Eco*RI
5'-GCGCGAATTCCAGGTCCAAATCAAGACAC-3'
5' location on MHV-68 genome: 70,994

IA3 & IA4 product: 1560bp
Target : MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temperature: 55°C, extension time: 2 minutes.

GST1n (sense)
*Nco*I
5'-CGGACCATGGTCATGTCCCCTATACTAGG-3'
5' location on pGEX-2T: 256

GST2e (antisense)
*Eco*RI
5'-GCGCGAATTCGGAACCAGATCCGATTTTG-3'
5' location on pGEX-2T: 926

GST1n & GST2e product: 689bp
Target: pGEX-2T
PCR conditions with *Taq* DNA polymerase: annealing temperature: 51°C, extension time: 1 minute.

MA1 (sense)
*Nco*I
5'-GGACCATGGGATTTCTGGGGAATCACAACTTAG-3'
5' location on MHV-68 genome: 69,520

MA2 (antisense)
*Xho*I
5' –GCTCTCGAGGGTAGAAATGTCTGGAACGG-3'
5' location on MHV-68 genome:70,826
Restriction site 5' end: *Xho*I

MA1 & MA2 product size: 1324bp
Target: MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temp: 50°C, extension time: 2 minutes.

MA2stop (antisense)
*Xho*I Stop
5'-CGCGCTCGAGTTAGGTAGAAATGTCTGGAACG G-3'
5' location on MHV-68 genome:70,824

IE1gp150 & MA2stop product size: 1391bp
Target: MHV-68 genome
PCR conditions with *Taq* DNA polymerase: annealing temperature: 51°C, extension time: 1.5 minutes.

CMV1(sense)
*Acc*I
5'- CGCGGTCGACGTTCATAGCCCATATATGGAG-3'
5' location on pEGFP-C1 plasmid (Clontech): 35

SV40(antisense)
*Acc*I
5'-GCGCGTCGACCGCGTTAAGATACATTGATGAG
5' location on pEGFP-C1 plasmid (Clontech): 1646

CMV1 & SV40 product size: 1631bp
Target: pEGFP-C1 (Clontech)
PCR conditions using *Pfu* DNA polymersase: annealing temperature: 45°C, extension time: 4 minutes.

RCVd (sense)
5'-GGACGCGTTCATCGATGATG-3'
5' location on MHV-68 genome: 69,165

SV40d (antisense)
5'-GCATTCTAGTTGTTGTCC-3'
5' Location on pEGFP-C1: 1598

RCVd & SV40d product size:626bp
Target: MHV-68 pRCVII generated recombinant virus genome
PCR conditions using *Taq* DNA polymerase: annealing temperature: 57°C, extension time 1 minute, 40 cycles.

PAG1 (sense)
5'-GTAGGATCCGTGAGAGTGTACACAAAGACGC-3' (sense)
5' location on MHV-68 genome:69,698

PAG2 (antisense)
5'-GGAGAATTCTCCTTTGGTTCAGC-3' (antisense)
5' location on MHV-68 genome:70,199

PAG1 & PAG2 product: 501bp
Target: MHV-68 genome (*gp150*)
PCR conditions using *Taq* DNA polymerase: anneal temperature: 57°C, extension time: 1 minute.

PAG11 (sense)
5' –CACCTCAGAACCAACTTC-3'
5' location on MHV-68 genome: 69,765

PAG12 (antisense)
5' –GTATCTGATGTGTCAGCAG-3'
5' location on MHV-68 genome: 70,130

PAG11 & PAG12 product: 367bp
PCR conditions using *Taq* DNA polymerase: anneal temperature: 49°C, extension time: 1 minute.

PAG11 & MA2
Product size: (a) 1059bp or (b) 2.68kbp
Target: (a) wild type MHV-68 or (b) recombinant virus I respectively.
PCR conditions using *Taq* DNA polymerase: annealing temperature: 50°C, extension time: 3 minutes.

PGB1 (sense)
*Xho*I *Bgl*II
5'-CGCGCTCGAGATCTTGGAGGTATATCAGAGAG-3'
5' location on MHV-68 genome: 16,240

PGB2 (antisense)
*Hin*dIII
5'-GCGCAAGCTTCCAACACTGGCTGAATCGG-3'
5' location on MHV-68 genome: 16,505

PGB1 & PGB2 product size: 286bp
Target: MHV-68 genome
PCR conditions using *Taq* DNA polymerase: annealing temperature: 53°C, extension time: 30 seconds.

PGB1 & IA4 product size: 1.8kbp
Target: pBabe/PgBgp150
PCR conditions using *Taq* DNA polymerase: annealing temperature 53°C, extension time: 2 minutes and 15 seconds.

Murine β-actin forward primer (β-AC1)
5'-TGTGATGGTGGGAATGGGTCAG-3'

Murine β-actin reverse primer (β-AC2)
5'-TTTGATGTCACGCACGATTTCC-3'

β-AC1 & β-AC2 product size: 514bp
PCR conditions using *Taq* DNA polymerase: annealing temperature: 59°C, extension time: 45 seconds.

HexaHistidine oligonucleotides

His1 (Sense)
*Bam*HI *Eco*RI
5'-CGCGGATCCCACCACCACCACCACCACTGAATTCGCG-3'

His2 (Antisense)
*Eco*RI *Bam*HI
5'-CGCGAATTCAGTGGTGGTGGTGGTGGTGGGATCCGCG-3'

Sequencing primers

Gp150 spanning primers

1I (antisense) – 5' location on MHV-68 genome: 69,601
5'-CTCTACTTGTAATACCCTC-3'
Annealing temperature: 49°C

3I (sense) - 5' location on MHV-68 genome: 69,767
5'-CCTCAGAACCAACTTCCC-3'
Annealing temperature: 51°C

6I (sense) - 5' location on MHV-68 genome: 70,069
5'-CAGGAAGATGAACCCACC-3'
Annealing temperature: 51°C

9I (sense) - 5' location on MHV-68 genome: 70,324
5'-GTTCCTCAACCAACTGCC-3'
Annealing temperature: 51°C

12I (sense) - 5' location on MHV-68 genome: 70679
5'-GTGGACCATCTATGACACC-3'
Annealing temperature: 53°C

PGBseq(sense)
5'-CTTGGAGGTATATCAGAGAG-3'
5' location on MHV-68 genome: 16,240
(putative gB promoter)

Plasmid primers

Ecdysone forward (sense)
5'-CTCTGAATACTTTCAACAAGTTAC-3'

BGH reverse (anti-sense)
5'-TAGAAGGCACAGTCGAGG-3'

T7-Promoter (sense)
5'-TAATACGACTCACTATAGGG-3'

SP6-Promoter (anti-sense)
5'-CGATTTAGGTGACACTATAG-3'

2.1.9 DNA Sequencing

Sequencing of plasmid DNA was performed by Ian Bennet within the Department of Veterinary Pathology using an LI-COR DNA sequencer 4000 (MWG-Biotech).

2.2.1 Restriction Enzyme Digestion of DNA

The restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Hin*dIII, *Nco*I, *Xho*I and *Acc*I were purchased from Gibco BRL and were used according to the manufacturers instructions in the supplied buffers. The standard reaction mix consisted of 1 unit of a restriction enzyme to 1μg of target DNA in the appropriate buffer (total volume ranging from 20 -50 μl) this was contained in 1.5ml microfuge tube and incubated in a 37°C water bath for 2 hours. Dnase free RNase, (1μl of 10mg/ml) was added to the reaction mix, prior to incubation, when the DNA target had significant RNA contamination.

2.2.2 Dephosphorylation of Exposed DNA Termini

The 5' phosphate of the cut ends of plasmid DNA following restriction digestion were dephosphorylated by the direct addition of 10 units of calf intestinal alkaline phosphatase (Boehringer) to the restriction digest reaction mix for a further 20 minute incubation at 37°C. The reaction was stopped by phenol/chloroform extraction as described in section 2.2.7.

2.2.3 Generation of Blunt Ended DNA

T4 DNA polymerase (Gibco BRL) was used to fill in the 3'-overhangs of pSP72/gp150 generated by *Acc*1 digestion. Following restriction digestion DNA was extracted with phenol/chloroform and precipitated with ethanol as described in

section 2.2.7. pSP72/gp150 DNA was then resuspended in a 100µl solution containing 10 units of T4 DNA polymerase, 1µl dNTP mix (Gibco BRL) (25mM of each nucleotide), 20µl T4 DNA polymerase buffer (Gibco BRL), 78µl H_2O and then incubated for 30mins at 37°C in a water bath.

2.2.4 DNA Ligation

Ligation of DNA fragments was carried out using T4 DNA ligase (Gibco BRL). Purified DNA fragments were mixed with the cleaved vector DNA at an approximate ratio of 5:1 respectively in ligase buffer (50mM Tris-HCl, pH7.6, 10mM $MgCl_2$, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000, Gibco BRL), 1 unit of T4 DNA ligase and 1mM ATP at a final volume of 20µl. This reaction mix was incubated at 4°C overnight.

2.2.5 Agarose Gel Electrophoresis

DNA samples, restriction enzyme digests and PCR products were mixed with 1/6 volume of gel loading buffer (H_2O containing 15% ficoll type 400, 0.25% bromophenol blue, 0.25% xylene cyanol and 0.25% orange G). A 1kbp DNA ladder (Gibco BRL), was used as a marker to determine the size of bands in kilobase pairs. Samples were loaded into a horizontal agarose gel made from 0.7% SeaKem LE agarose (Flowgen) dissolved in TAE buffer (40mM Tris-acetate, pH 7.8 and 1mM EDTA) with 2.5µg/ml ethidium bromide. The gel was submerged in a Bio-Rad mini sub tank containing TAE buffer and samples electrophoresed at a constant 50 volts. DNA bands were visualised using a UV transilluminator.

2.2.6 Isolation of DNA Fragments from Agarose Gels

The DNA for isolation was visualised in an agarose gel on a UV transilluminator and a sterile scalpel blade was used to cut the required band from the gel. Dialysis tubing, pore size of a 12,000 MW cut off point (BDH), was prepared by boiling in H_2O plus 1 mM EDTA for 10 minutes. The tubing was rinsed with H_2O and the piece of gel placed into the tubing with 200µl elution buffer (10mM Tris-HCl pH 7.9, 1mM EDTA plus 1% gelatin). The dialysis tubing was clamped at each end and placed in an electrophoresis tank with TAE buffer, the DNA was electrophoresed

from the gel into the elution buffer for 1 hour at 70 volts. The elution buffer, containing the DNA was transferred to a microfuge tube and phenol/chloroform extraction followed by ethanol precipitation was done as described in section 2.2.7.

2.2.7 Phenol/Chloroform Extraction and Ethanol Precipitation of DNA

To remove protein contaminants from DNA solutions phenol/chloroform extraction was performed. To a DNA solution an equal volume of phenol/chloroform solution (phenol, chloroform and isoamyl alcohol, ratio of 25:24:1 respectively, pH7.5) was added, mixed and then centrifuged for 30 seconds at 10,000 *x g*. The upper phase containing the DNA was removed and transferred to a new tube containing an equal volume of chloroform. The tube was mixed and then centrifuged for 30 seconds at 10,000 *x g*. The upper phase was removed and added to a new tube containing ½ volume of 7.5M ammonium acetate and then 3 volumes of ethanol were added. The tube was incubated at -20°C for 1 hour to allow the DNA to precipitate. The tube was spun for 10 minutes at 10,000 *x g* to pellet the DNA. The supernatant was discarded and the pellet washed with 200µl of 70% ethanol. The tube was spun as before and then the pelleted DNA was resuspended in TE buffer (10mM Tris-HCl pH 7.9, 1mM EDTA).

2.2.8 Purification of DNA Fragments Generated by PCR

To purify PCR cloned DNA fragments a wizard column PCR prep kit (Promega) was used according to manufacturers instructions.

2.2.9 Extraction of Viral DNA

For the extraction of viral/cellular DNA from mammalian cells, ~$5x10^7$ cells in a T175 Nunc culture flask 5 days following infection with MHV-68 at 0.01 MOI were washed twice in PBS. Cells were resuspended at 5 x 10^7 cells/ml in TE and transferred to a 15ml polypropylene tube (Falcon). To the cell suspension, 10 volumes of extraction buffer (0.1M EDTA, 0.5% SDS, 0.2M Tris pH 8.0), containing 20µg/ml RNase (Sigma) was added. Following incubation of the tube for 1 hour at 37°C, proteinase K (Sigma) was added at a final concentration of 100µg/ml and incubation was done overnight at 56°C. Phenol/Chloroform extraction was done

three times followed by one chloroform extraction. For each extraction the tube was rotated for 15 minutes at RT and spun at 2500 *x g* for 15 minutes each time. Ethanol precipitation of the DNA was done at room temperature.

For small scale preparations of DNA from wells of 96 well plates up to T25 Nunc tissue culture flasks of cell cultures a Qiamp DNA extraction kit (Qiagen) was used.

2.3.1 Radioactive Labelling of DNA fragments

DNA radiolabelled with the isotope ^{32}P was used to probe Southern and northern blots. A random primed DNA labelling kit (Boehringer Mannheim) was used to generate probes as follows. To denature target DNA (either PCR products or DNA fragments eluted from agarose gels), 25ng in 9µl H_2O was heated at 95° C for 10 minutes and then cooled on ice. Subsequently, 1µl of each dNTP; dATP, dGTP and dTTP, 2µl hexanucleotide reaction mix (Boehringer Mannheim), 5 µl [$\alpha^{32}P$] dCTP (50µCi) (Amersham) and 1µl/2units Klenow enzyme (Boehringer Mannheim) was added to the DNA and the tube incubated in a 37°C water bath for 35 minutes. TE buffer was then added to increase the volume of the mix to 100µl. The radiolabelled probe was then purified from the unincorporated dNTPs using a sephade*x* *g*-50 NICK^{tm} column (Pharmacia Biotech) resulting in 500µl final probe.

2.3.2 Southern Blotting

For Southern blotting, 5µg of each DNA sample was electrophoresed in a standard 0.7% TAE/agarose gel as above. Subsequently, the gel was immersed in gel soak 1 (0.5 M NaOH, 1 M NaCl) for 20 minutes two times, to denature the DNA. The gel was then soaked 2 times in a neutralisation buffer (0.5M Tris HCl, pH7.5, 1.5 M NaCl) for 20 minutes. The gel was rinsed in H_2O and transferred to a tank for capillary transfer of the DNA onto a nylon membrane (Micron Separations Inc). The gel was placed on a platform directly on top of three sheets of 3mm paper with ends immersed in 10x SSC (1.5M NaCl, 0.15M NaCitrate, pH 7.0) in wells. A nylon membrane was placed on top of the gel followed by 2 sheets of 3mm paper, many layers of absorbent paper towels and a heavy weight. The gel was blotted overnight. To cross link the transferred DNA to the nylon, the membrane was placed in a UV

Stratalinker™ 1800 (Stratagene) and pulsed with UV light for 30 seconds. To stain the markers, the marker lane was cut off and immersed in 1M acetic acid for 10 minutes followed by 10 minutes in methylene blue (0.2% methylene blue, 0.4M acetic acid, 0.4M sodium acetate) and rinsed in H_2O. Blots were stored at –70°C.

2.3.3 Probing Southern Blots

Prior to probing, Southern blots were incubated in hybridisation solution (6 x SSC plus 5% non fat dried milk) at 68°C for 2 hours. The required radiolabelled probe generated as in section 2.3.1 was denatured by the addition 0.1 volume of 1 M NaOH and incubated at 37°C for 5 minutes. The blot was then incubated at 68°C, overnight, in fresh hybridisation solution, to which the denatured probe had been added. Excess probe was washed from the blot by a series of increasingly stringent washes i.e. 2 x 15 minutes in 5 x SSC plus 0.5% SDS (sodium dodecyl sulphate) at RT, 2 x 15 minutes in 1 x SSC plus 0.5% SDS at 37°C and 3 x 15 minutes in 0.1 x SSC plus 1% SDS at 55 – 65°C. Between each wash the radioactivity level on the blot was monitored using a Geiger counter. After the final wash the nylon membrane was wrapped in Saran wrap (Dow, USA) and loaded into a film cassette with X-OMAT XAR-5 Kodak film and a DuPont Cronex Lightning Plus intensifying screen (Sigma). Film cassettes were placed at –70°C. Exposure times varied from 2 hours to two weeks. Prior to developing the film, cassettes were fully thawed. To remove bound probe from blots in order to re-probe the blot with different probes the blots were subject to stringent washes in boiling water plus 1% sodium dodecyl sulphate. Stripped blots were exposed to film overnight to monitor removal of radioactive probe.

2.3.4 Cytoplasmic RNA Extraction

For the extraction of cytoplasmic RNA from mammalian cells, cells were harvested from a confluent T175 Nunc tissue culture flask and then washed twice in PBS. Following this, cells were resuspended in 1 ml of ice cold Tris saline (25mM Tris, pH 7.4, 130mM NaCl and 5mM KCl). The suspension was transferred to a 1.5 ml microfuge tube, spun at 3,000 *x g* for 30 seconds and the cells resuspended in 400µl ice cold Tris saline. To this, 100µl of Igepal buffer (1% Igepal CA-630, 0.5%

sodium deoxycholate and 0.01% dextran sulphate in Tris saline) was added and the tube inverted ten times followed by centrifugation at 3000 *x g* for 30 seconds. The supernatant was transferred to a microfuge tube containing 500µl phenol/chloroform (1:1), then 25µl 20% SDS and 15µl 5M NaCl was added and the tube vortexed for 30 seconds. Following centrifugation at 10,000 *x g* for 2 minutes the aqueous phase was transferred to a new tube with 500 µl phenol/chloroform and then vortexed and spun as before. This phenol extraction was repeated 3 times until the interface was clear. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the recovered aqueous phase and the tube was vortexed and spun as before in order to extract the phenol. The aqueous phase was transferred to a tube containing 1ml of ethanol which was then incubated at –20°C from 30 minutes to overnight. Following centrifugation at 10,000 *x g* for 20 minutes at 4°C the RNA pellet was washed in 80% ethanol and spun at 10,000 *x g* for 20 minutes. The pellet was allowed to dry and then resuspended in 50µl diH_2O. To quantify the RNA the optical density of 1µl diluted in 500µl H_2O was measured at 260/280nm. To check the quality of the RNA, 1-3µg RNA was mixed with 1/6 volume of gel loading buffer (50% glycerol, 1mM EDTA [pH8.0] and 0.25% bromophenol blue) and loaded in an electrophoresis gel consisting of 0.8% SeaKem LE agarose (Flowgen) dissolved in TBE (89mM Tris-borate, pH7.5, and 2mM EDTA) containing 2.5µg/ml ethidium bromide. The gel was submerged in a Bio-Rad mini sub tank containing TBE buffer and the samples electrophoresed at a constant 50 volts. Ribosomal RNA bands were visualised using a UV transilluminator.

2.3.5 mRNA extraction for RT-PCR

For extraction of RNA from monolayers in six well plates, media was removed and wells were washed once with ice cold PBS. Using the plunger of a 1 ml syringe the cells (~10^6/well) were scraped into 1 ml PBS and transferred to a 1.5 ml microfuge tube. To extract the mRNA a mRNA capture kit (Boehringer Mannheim) was used according o manufacturers instructions. Cells were pelleted by centrifugation at 250 *x g* and resuspended in 200µl of ice cold lysis buffer. The DNA was sheared by passing the sample through a 21 gauge needle six times. A solution containing 0.25 nmol of biotin-labeled oligo$(dT)_{20}$, 4µl, was added to the sample and the mix was

incubated for 5 minutes at 37°C. For immobilisation of the poly(A^+) RNA, 50µl of the mix was added to a strepavidin-coated 0.2 ml PCR tube and incubated for 3 minutes at 37°C. The mix was removed from the tube and washed three times using 200µl of washing buffer. The captured mRNA was then used directly for reverse transcription and amplification by PCR in the tube as described in section 2.1.5.

2.3.6 Northern Blotting

For northern blotting, RNA samples were prepared by mixing 5.5µl (5µg) of RNA with 1µl 10 x MOPS (0.2M 3-[N-Morpholino] propanesulphonic acid [pH7.0], 80mM sodium acetate and 10mM EDTA [pH 8.0]), 3.5µl formaldehyde and 10µl formamide). For denaturation of the RNA, the RNA solution was heated at 65°C for 15 minutes and then cooled on ice. Following the addition of 2µl of RNA loading buffer (50% glycerol, 1mM EDTA [pH8.0] and 0.25% bromophenol blue) 10µl was loaded onto a 1.2% agarose gel (1.2% 'SeaKem' agar, 2.2M formaldehyde, 1 x MOPS, 1µg/ml ethidium bromide). The gel was electrophoresed overnight at 35 volts in a Biorad electrophoresis gel tank in which the running buffer (1x MOPS) was continuously recirculated.

The gel was then blotted by capillary transfer onto a nylon membrane (Micro Separations Inc.). To cross link the RNA to membrane, the membrane was placed in a UV Stratalinker™ 1800 (Stratagene) and pulsed with UV light for 30 seconds. To stain the markers, the marker lane was cut off and immersed in 1M acetic acid for 10 minutes followed by 10 minutes in methylene blue (0.2% methylene blue, 0.4M acetic acid, 0.4M sodium acetate) and rinsed in H_2O. Blots were stored at –70°C.

2.3.7 Probing Northern Blots

Reagents:

50x Denhardt's Solution:	5% Ficoll (MW 400), 5% polyvinylpyrrolidone (MW 360,000) and 5% BSA in H_2O
20 x SSPE:	3 M NaCl, 0.2M Na_2PO_4, 0.02M EDTA in H_2O, pH7.4.

Salmon Sperm DNA (Sigma):	5mg/ml DNA, boiled for 5 minutes and sheared by passing through a 21-gauge needle
Prehybridisation buffer:	5 x SSPE, 5 x Denhardt's, 50% formamide, 0.5% SDS and 100μg/ml salmon sperm DNA.
Hybridisation buffer:	5 x SSPE, 5 x Denhardt's, 50% formamide, 10% dextran sulphate. 0.5% SDS and 200μg/ml salmon sperm DNA.

Prior to probing with radiolabelled DNA probes, northern blots were incubated in prehybridisation buffer for 2 hours at 45°C. The required radiolabelled probe generated as in section 2.3.1 was denatured by the addition of 0.1 volume of 1 M NaOH and incubated at 37°C for 5 minutes. The blot was then incubated overnight, at 45°C, in hybridisation solution, to which the denatured probe had been added. To remove excess probe the blot was subjected to a series of increasingly stringent washes i.e. 2 x 15 minutes in 5 x SSPE, 0.5% SDS at RT, 2 x 15 minutes in 1 x SSPE, 0.5% SDS at 37°C and 3 x 15 minutes 0.1% SSPE, 1% SDS at 55-65°C. The blot was then wrapped in saran wrap and placed in a film cassette with X-OMAT Kodak film and a DuPont Cronex Lightning Plus intensifying screen (Sigma).

BACTERIAL WORK

2.3.8 Bacterial Strains

Commercial competent *Escherichia coli* (*E. coli*) (Epicurian Coli XL1-Blue cloning-grade competent cells, Stratagene) were used for plasmid cloning and for protein expression from vector pGEX-2T. BL21 (DE3) *E. coli* (Novagen), containing the gene for T7 RNA polymerase under the control of the *lacUV5* promoter which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG), were used for protein expression from pET22b based vectors.

2.3.9 Heat Shock Transformation of Bacteria with Plasmid DNA

Frozen competent bacteria were thawed on ice. Competent cells, 100µl were transferred into a prechilled Falcon 2059 polypropylene tube. β-mercaptoethanol (Invitrogen), 1.7µl, was added to the 100µl bacteria (giving a 25mM final concentration) and the tube was incubated on ice for 10 minutes swirling every two minutes. Ligation mix (amount of DNA ranging between 0.1 - 50ng), 1µl, was added to the cells which were incubated on ice for a further 30mins. The tube was then placed in a 42°C water bath for 45 seconds and returned to ice for 2 minutes. LB medium (Luria Bertani medium: 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl per litre at pH 7.0), 900µl, was then added to the tube and the bacteria were incubated at 37°C shaking for 1 hour. Transformed bacteria, 100 - 900µl were removed and spread onto bacterial plates. Bacterial agar plates were made by the addition of bacto-tryptone agar at 15g/L to LB medium prior to autoclaving, before pouring plates the selection agent consisting of either 100µg/ml ampicillin (LB amp^+) or 50µg/ml carbenicillin was added to the LB. The LB agar plates were incubated at 37°C overnight following application of bacterial samples.

When growing and selecting bacteria transformed with pVgRXR the selective antibiotic Zeocin™ was used at 25µg/ml in low salt LB medium. Low salt LB medium consisted of 10g Tryptone, 5g NaCl and 5g yeast extract made up to a litre with H_2O.

2.4.1 Colony Transformation of BL21(DE3) Bacteria

From freshly streaked BL21(DE3) bacteria, 2-4 colonies were picked from an LB agar plate containing 10mM $MgCl_2$. The colonies were dispersed in 200µl of transformation buffer; TFB (H_2O containing 100mM KCl, 45 mM $MnCl.4H_2O$, 10mM $CaCl_2.2H_2O$, 3mM $HACoCl_3$ and 10 mM K-MES (2[morpholino]ethone sulphonic acid plus KOH to pH to 6.3) in a Falcon 2059 polypropylene tube. The tube was incubated on ice for 15-30 minutes. DND solution, 7µl, consisting of 90% (v/v) dimethyl sulfoxide (DMSO), 10mM potassium acetate and 1M dithiothreitol (DTT) was added to the tube. The tube was swirled and then incubated on ice for

10-15 minutes. Another 7µl of DND was added to the tube which was then swirled and incubated on ice for 10-20 minutes. Plasmid DNA solution, 2µl (~50ng), was added to the tube and then incubated on ice for 30 minutes. To heat shock the cells the tube was placed in a 42°C water bath for 90 seconds and then chilled on ice for 2 minutes. SOC medium (LB containing 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose), 800µl, was added to the tube and incubated at 37°C with shaking for 1 hour. Transformed bacteria, 100-900µl were plated onto LB amp^+ agar plates and incubated at 37°C overnight.

2.4.2 Glycerol Stocks

For long term storage of plasmid containing bacteria, glycerol stocks were made. Glycerol, 0.15 volumes was added to 0.85 volumes of a growing liquid culture. Aliquots of 1ml of this mix in 1.5 ml microfuge tubes were flash frozen by placing in solid CO_2/ethanol and stored at –70°C.

2.4.3 Small Scale Purification of Plasmid DNA from Bacteria

Small scale plasmid preparations (minipreps) to isolate plasmid DNA from transformed bacteria were done by using an alkaline lysis method. Colonies were picked from freshly streaked plates of transformed bacteria and each was inoculated into individual 10ml aliquots of LB medium containing 100µg/ml ampicillin (LBA medium) in 25ml plastic universal tube (Sterilin). The tubes were incubated overnight at 37°C with shaking. The bacteria were pelleted by centrifugation (4000 *x g*, 5 minutes), resuspended in 200µl glucose solution (50mM glucose, 25mM Tris-HCl (pH8.0) and 10mM EDTA) plus 5mg/ml hen egg lysozyme (Sigma), transferred to 1.5ml microfuge tubes and incubated for 5 minutes at room temperature (RT). All subsequent incubations were carried out on ice. Lysis solution, (0.2M NaOH, 1% SDS), 400µl, was added to each tube and mixed gently. Following a 5 minute incubation, cold neutralisation solution (3M potassium acetate, 2M acetic acid), 300µl, was added to each tube, mixed vigorously and incubated for 15 minutes. After centrifugation of the tubes at 10,000 *x g* for 5 minutes the supernatants were transferred to new microfuge tubes containing 500µl propan-2-ol and incubated for 15 minutes. The nucleic acid was pelleted by centrifugation of the tubes (10,000 *x g*,

5 minutes). The supernatant of each tube was decanted off and the pellet was allowed to dry before resuspending in 200µl TE buffer. 100µl of 7.5 M ammonium acetate was added to each tube followed by a 15 minute incubation.. The tubes were centrifuged at 10,000 *x g* for 5 minutes and each supernatant was transferred to a new microfuge tube containing 600µl ethanol and incubated for 1 hour at -20°C to precipitate the DNA. To pellet the precipitated DNA the tubes were spun for 5 minutes at 10,000 *x g*. DNA pellets were each suspended in 20-50µl TE buffer.

To yield larger quantities of plasmid DNA large scale plasmid preparations (maxipreps) were done on 400ml LBA medium bacterial cultures using either the caesium/chloride gradient method according to Sambrook *et al*, 1989 as described below or a Qiagen maxi prep column.

2.4.4 Large Scale Preparation of Plasmid DNA from *E. coli*

In order to grow larger volumes of bacterial cultures, 10 ml of an overnight culture was added to 400ml LBA in a 2 litre flask which was then incubated at 37°C, shaking overnight. The bacteria were pelleted by centrifugation at 7000 *x g* for 5 minutes. The pellet was resuspended in 20ml of glucose solution, and incubated for 10 minutes at room temperature. All subsequent incubations were done on ice. Lysis solution (as for small scale DNA preparation), 40 ml, was added to the tube, mixed gently and incubated for 5 minutes. Following this, 20 ml of neutralisation solution was added, and the tube incubated for 15 minutes. After centrifugation, as before, the supernatant was passed through a gauze filter, 0.6 volumes of propan-2-ol added. Following a 30 minute incubation the tube was spun as before, the supernatant discarded and the pellet allowed to dry. The pellet was then resuspended in 6 ml TE to which 3 ml 7.5 M ammonium acetate was added and then incubated for 30 minutes in a 30ml glass Corex tube. The tube was spun at 10,000 *x g* for 10 minutes and the supernatant transferred to a new tube. DNA was precipitated by the addition of 18 ml ice cold ethanol and incubation at –20°C for 1 hour. The tube was then spun at 10,000 *x g* for 10 minutes and the pellet resuspended in TE/CsCl 1:1 (v/w). This solution was then loaded into a 36ml Quick seal^{tm} ultraclear polyallomer tube (Beckman), and 700µl of ethidium bromide (5mg/ml) was added to a full tube i.e.

36ml TE/CsCl. Tubes were heat sealed, placed in a Vti50 rotor and spun at 242,000 *x g* in a Beckman L8-M ultracentifuge for 18 hours. The resultant DNA bands in the CsCl gradient were visualised using the UV transilluminator. The supercoiled plasmid band was drawn off using a wide bore syringe. To remove the ethidium bromide from the harvested DNA an equal volume of CsCl/TE saturated butanol was added and the tube inverted to mix. The lower layer, containing the DNA, was drawn off to a new tube. Again, an equal volume of CsCl/TE saturated butanol was added and the tube inverted and the lower layer pipetted off. This process was repeated two more times until no pink colour from contaminating ethidium bromide could be seen. The CsCl was then removed from the DNA by dialysing the solution against several changes of TE, overnight at 4°C. The DNA was precipitated by the addition of 0.5 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol followed by incubation for 1 hour at –20°C. The tube was spun at 10,000 *x g* for ten minutes, the pellet washed with 70% ethanol, the tube spun as before and the pellet resuspended in 500µl TE.

2.4.5 Protein Expression and Purification

For expression of fusion proteins, BL21 (DE3) bacteria containing the pET22b based vector plasmids were grown in 100ml LB medium with 50µg/ml carbenicillin on a shaker at 37°C. When the culture reached an optical density (OD) of approximately 0.6 at 600nm, isopropyl β-D-thiogalactopyranoside (IPTG) was added (1mM final concentration) to induce fusion protein expression. The culture was incubated for a further 2.5 hours. To purify the periplasmic fraction of the bacteria, the cells were pelleted by centrifugation (5 minutes at 500 *x g*) and resuspended in 0.4 culture volumes of 30mM Tris-HCl pH8.0, 20% sucrose, 1% (v/v) aprotinin (Sigma) and 1mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The suspension was incubated at room temperature for 10 minutes with shaking and then centrifuged as before. The pellet was resuspended in 0.4ml volumes of ice cold 5mM $MgSO_4$ and incubated on ice for 10 minutes with shaking. The suspension was centrifuged as before and the supernatant retained (the periplasmic fraction). The His tagged fusion protein was extracted by affinity purification using Ni^{2+} charged resin beads (Novagen) according to manufacturers instructions. Briefly, prior to incubation the

HisBind resin was prepared by sequential washes in 3 volumes of deionised water, 5 volumes of charge buffer (50mM $NiSO_4$) and 3 volumes of binding buffer (5mM imidazole, 500mM NaCl, 20mM Tris-HCl pH7.9). All steps were carried out at 4°C. The prepared periplasmic fraction (approximately 35ml from a 100ml culture) was mixed with 5ml 8x concentrated binding buffer (40mM imidazole, 4M NaCl, 160mM Tris-HCl pH7.9) to give a final concentration of 1x binding buffer. The mix was added to 5ml (settled bed volume) Ni^{2+} charged HisBind metal chelation resin in a 50 ml Falcon tube and incubated on a rotator for 2 hours. Resin was settled by centrifugation for 3 minutes at 500 *x g*, brake off. The resin was subjected to a series of 15 minute washes on a rotator including 10 volumes binding buffer, 6 volumes wash buffer (60mM imidazole, 50mM NaCl, 20mM Tris-HCl, pH7.9) and 6 volumes elute buffer (1M imidazole, 500mM NaCl, 20mM Tris-HCl, pH7.9). The eluate (elute buffer post incubation with resin) was retained and dialysed against several changes of PBS overnight. Concentration of the fusion protein was achieved by filter centrifugation of the eluate at 500 *x g* using Centriplus concentrators (Amicon) pore size of 30 (molecular weight cut off point of 30,000). Protein samples were stored at –20°C. Fractions from each stage of the purification process were routinely retained and analysed by SDS-PAGE. The Ni^{2+} charged resin was stripped by incubation in strip buffer (100mM EDTA, 500nm NaCl, 20mM Tris-HCl pH7.9).

For expression of fusion protein from XL1 blue *E. Coli* containing pGEX-2T based vector plasmids bacteria were grown in 100ml LB medium containing 100μg/ml ampicillin. Induction was performed as above. Bacteria were pelleted by centrifugation at 5000 *x g* for 5 minutes and resuspended in 4ml binding buffer. The suspension, in a tube on ice, was probe sonicated using an ultrasonic generator at 50% power (Dawe Instruments Ltd.) in a series of three 15 second bursts. The bacterial lysate was centrifuged at 10,000 *x g* for 20 minutes at 4°C. The supernatant (soluble fraction) was retained and applied to 2.5ml Ni^{2+} charged HisBind resin. Purification and concentration of the His tagged fusion protein proceeded by the same method used for purification of His tagged fusion proteins from the periplasmic fraction as described above.

2.4.6 Determination of Protein Concentration

Protein concentration was measured using a Cecil CE 2041 spectrophotometer by reading the optical density at 280nm of each protein sample diluted in PBS contained in a glass cuvette. Protein concentration was calculated according to Beer-Lambert's law (Stoscheck, 1990):

$A_{280} = \varepsilon CL$

Where A_{280} is the absorbance at 280nm, ε is the molar extinction coefficient of the protein at 280nm, C is the molar concentration of the sample and L is the path length in cm. For glutathione-S-transferase an absorbance of 1 at 280nm = 0.5mg/ml (Smith & Johnson, 1988).

2.4.7 Biotinylation of Protein

Approximately 1 mg/ml of protein was dialysed in 0.1M $NaHCO_3$, pH 8.3-8.4 overnight at 4°C. To every 100µl of dialysed protein solution 50µl of biotin 1mg/ml in H_2O was added and then incubated at 4°C overnight. The protein solution was then dialysed in phosphate buffered saline (PBS: 80mg/ml NaCl, 2mg/ml KCl, 2mg/ml KH_2PO_4, 11.05mg/ml Na_2HPO_4, pH 7.2) to remove excess biotin.

2.4.8 SDS-PAGE

In preparation for sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), 2 - 3 x 10^7 eukaryotic cells per ml in a microfuge tube on ice were subjected to probe sonification by a series of 5 second bursts using an ultrasonic generator at 50% power (Dawe Instruments Ltd.). Lysates were suspended in an equal volume of 2 x SDS-PAGE sample buffer (100mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.02%bromophenol blue, 200mM dithiothreitol) and samples were heated to 95°C for 5 minutes. Cell lysate, 10µl, was loaded per well of SDS-PAGE gel, equivalent to approximately 1.5 x 10^5 cells. Bacterial cells were pelleted from 10ml culture media by centrifugation (4000 *x g*, 5 minutes), resuspended in 500µl PBS in a microfuge tube on ice and were subjected to probe sonification by a series of 5 second bursts using an ultrasonic generator at 50% power (Dawe Instruments

Ltd.). The bacterial cell lysate was mixed with an equal volume of 2x SDS-PAGE sample buffer and heated for 5 minutes at 95°C. In order to prepare crude soluble and insoluble fractions of the bacteria, cells were pelleted from 10ml of culture media by centrifugation (4000 *x g*, 5 minutes) and resuspended in 500µl 50mM Tris-HCl pH8.0, 2mM EDTA in a microfuge tube. Hen egg lysozyme to a concentration of 100µg/ml and then Triton X –100 to a final concentration of 0.1% was added and the tube incubated at 30°C for 15 minutes. The microfuge tube was placed on ice and subjected to probe sonication by a series of 5 second bursts using an ultrasonic generator at 50% power (Dawe Instruments Ltd.). The tube was then centrifuged at 12,000 *x g* for 15 miuntes at 4°C. The supernatant contains the soluble proteins to which an equal volume of 2 x SDS sample buffer was added. The pellet, the insoluble fraction was resuspended in 1 x SDS sample buffer.

Samples (10µl) were electrophoresed using a vertical slab gel system ('Dual mini slab' Atto). The main separating gel was composed of 7.5% acrylamide:bisacrylamide (37.5:1) (National Diagnostics), 375mM Tris-HCl, pH8.8, 0.1% SDS, 0.7mg/ml ammonium persulphate and 0.08% TEMED (Sigma). The percentage of acrylamide:bisacrylamide in the main separating gel was varied from 5% to 14% depending on the samples analysed. The stacking gel was composed of 5% acrylamide (5% acrylamide:bisacrylamide, 125mMTris-HCl, pH 6.8, 0.1% SDS, 0.8mg/ml ammonium persulphate and 0.2% TEMED (Sigma)). The molecular weight protein markers used were prestained 'Rainbow' markers (Amersham) or Biorad prestained protein markers. Gels were electrophoresed at a constant current of 15-20mA per gel for approximately 2 hours. The process was stopped once the dye reached the bottom of the gel.

2.4.9 Staining SDS-PAGE Gels with Coomassie blue

Proteins electrophoresed through a polyacrylamide gel were visualised by incubating the gel with Coomassie blue (0.25% Coomassie brilliant blue R250, 10% acetic acid, 45% methanol) overnight and then incubating in a series of changes of excess destain (10% acetic acid, 20% methanol) for 2 hours to overnight until the background cleared. Gels were dried using a Biorad gel dryer for 2 hours at 80°C.

2.5.1 SDS-PAGE of Radiolabelled Samples

In order to enhance autoradiography, SDS-PAGE gels containing ^{35}S labeled samples were impregnated with Enhance (Dupont). Following electrophoresis gels were first soaked in protein fixer solution (10% glacial acetic acid (v/v), 30% methanol (v/v)) for 1 hour and then immersed in Enhance for 1 hour. Subsequently gels were incubated in an excess of water, for 30 minutes, to precipitate the fluorescent material in the gel. Gels were then dried as above and exposed to X-OMAT XAR-5 autoradiographic film.

2.5.2 Western Blotting

Proteins separated in a SDS-PAGE gel were transferred to a 0.45μm pore Immobilon-P membrane (Millipore) by electrophoresis in transfer buffer (30mM Tris, 200mM glycine, 20% methanol) using a Bio-Rad mini transfer blotting kit at 250mA for 60 minutes. After transfer the membrane was incubated in blocking buffer (50mM Tris, pH 7.4, 150mM NaCl (TBS) plus 5% powdered milk (Marvel) and 5% normal pig serum) for 60 minutes at RT. The membrane was then incubated in primary antibody diluted (dilutions used are displayed in section 2.5.4) in the blocking buffer for 2 hours at RT or overnight at 4°C. The blot was washed 3 times in TBS plus 0.2% Tween. The secondary antibody conjugated to alkaline phosphatase diluted 1:1000 in blocking buffer was added to the blot and incubated for a further hour at RT. The blot was washed 5 times with TBS, 0.2%Tween and then once in TBS. The blot was developed by soaking with nitro blue tetrazonium and 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) (1 Sigma tablet per 10ml H_2O). The reaction was stopped by the addition of excess H_2O.

2.5.3 Far-western/VOPBA –Virus Overlay Protein Binding Assay

To assay the binding of gp150-His fusion protein to proteins in a western blot, the membrane was incubated in blocking buffer (as for section 2.5.2 above) for 45 minutes at room temperature. After the initial blocking stage the fusion protein was diluted in blocking buffer and applied to the blot for 30 minutes to 2 hours at room RT or overnight at 4°C. The membrane was then washed and primary and secondary antibody steps followed as above. Alternatively, if a biotinylated fusion protein was

used an avidin conjugated to alkaline phosphatase Vectastain ABC-AP kit (Vector Labs) was used according to manufacturers instructions to detect binding. An alternative blocking buffer used contained 0.1% bovine serum albumin in TBS. To assay the binding of virus to proteins in a western blot the culture supernatant of BHK cells infected with MHV-68 (5 days post infection) was incubated with the blot or for higher concentrations of virus preparations as prepared in section 2.6.8 were diluted in blocking buffer and applied to blots for 30 minutes to 2 hours at room RT or overnight at 4°C. The membrane was then washed and primary and secondary antibody steps followed as for section 2.5.2 above.

2.5.4 Antibodies

Antibody	Working Dilution	Reference/ Source
Polyclonal rabbit anti-gp150-GST	1:500 WB, IS 1:250 FACS, ELISA	Stewart *et al.*, 1996
Polyclonal rabbit anti-MHV-68	1:500 WB IS	Sunil-Chandra *et al.*, 1992a
Polyclonal swine anti-rabbit IgG AP	1:1000 WB	Dako
Monoclonal mouse anti-His(C-term)	1:5000 WB 1:50 FACS	Invitrogen
Monoclonal rat anti-CD4	Neat FACS	In House
Monoclonal rat anti-CD8	Neat FACS	In House
Polyclonal goat anti-rat IgG FITC	1:500 FACS	Serotec
Polyclonal goat anti-rat IgG R PE	1:10 FACS	Serotec
Polyclonal goat anti-mouse IgG FITC	1: 50 FACS	Serotec
Polyclonal goat anti-mouse IgM biotinylated	1:100 FACS	Serolab
Polyclonal goat anti-rabbit IgG biotinylated	1:500	Serotec
Strepavidin-FITC	1:100 FACS	Serotec
Monoclonal rat-anti mouse CD19 (B cell marker)	1:200 FACS	Pharmingen
Polyclonal rabbit anti-mouse Ig HPX	1:2000 ELISA	Dako
Polyclonal rabbit-anti-mouse IgG/A/M HPX	1:2000 ELISA	Serotec
Polyclonal rabbit anti-mouse IgG2a	1:1000 ELISA	Serotec
Polyclonal rabbit anti-mouse IgG2b HPX	1:500 ELISA	Serotec
Polyclonal rabbit anti-mouse IgG1	1:500 ELISA	Serotec
Polyclonal swine anti-rabbit IgG FITC	1:30 FACS IF	Dako

Table 2.2. Antibodies and Immunodetection Reagents. Conjugates: FITC – fluorescine isothiocyanate, R-PE – R-Phycoerythrin, HPX – horseradish peroxidase, AP-alkaline phosphatase, GST – glutathione-S-transferase. FACS – fluorescence associated cell sorting, WB – Western blots, IF – Immunofluorescence, IS – Immunostaining, ELISA – Enzyme linked immunosorbent assay. In house normal rabbit, pig, goat and mouse sera were also used dilutions are indicated in the text.

EUKARYOTIC CELL WORK

2.5.5 Cell Lines

Cell Lines	Properties
Baby Hamster Kidney cell line (BHK-21)	An immortal fibroblastoid cell line (Stoker & Macpherson, 1961)
COS (COS-1)	SV40 transformed fibroblastoid cell line (Gluzman, 1981)
S11	Mouse derived B lymphoma cell line positive for MHV-68 episomal DNA (Usherwood *et al.*, 1996c)
NS0	Non-immunoglobulin secreting mouse myeloma cell line (Kholer and Milstein, 1976).
A20	Mouse B cell lymphoma cell line (Kim *et al.*, 1979)
EL4	A C57BL/6 murine T lymphoma cell line (Ralph, 1973)
C127 (ATCC CRL 1616)	Mouse epithelial cell line derived from a mammary carcinoma (Lowy *et al.*, 1978)
CV-1	African green monkey kidney cells, fibroblastoid (Jensen *et al.*, 1964)
S31	Mouse derived B lymphoma cell line (Usherwood *et al.*,1996c)

Table 2.3. Cell Lines

2.5.6 Tissue culture

Baby hamster kidney cells (BHK-21) were grown in T175 flasks (Nunc) seeded at 2 X 10^6 cells with BHK growth medium (Glasgow modified Eagle medium (GMEM) (Gibco BRL) supplemented with 10% (v/v) Tryptose phosphate broth (TPB) (Gibco BRL) and 10% (v/v) new born calf serum (NBCS) (Harlan Sera Lab), 70μg/ml penicillin (Merck BDH), 10μg/ml streptomycin (Sigma) and 2mM L-glutamine (Merck BDH)).

CV-1, COS-1 and C127 cells were grown in T175 flasks (Nunc) seeded at 2 x 10^6 with Dulbecco's modified Eagle medium (DMEM) (Gibco BRL) containing 10% Foetal calf serum (FCS) (Globepharm), 70μg/ml penicillin, 10μg/ml streptomycin and 2mM L-glutamine.

To passage adherent cell types (BHK, C127, COS-1 and CV-1 cells) monolayers were harvested by washing with 5 ml of versene solution (0.02% versene in sterile

PBS containing 200μg/ml ethylenediaminetetra acetic acid (EDTA) and 1% phenol red, pH 7.1-7.3) and then incubating with 5 ml of Trypsin/EDTA solution (tissue culture grade, Life Technologies, Paisley) until monolayers lifted from plastic. To dislodge cells and break up clumps flasks were tapped and cells were pipetted up and down. Trypsinisation was stopped by the addition of 15 ml growth medium. The cell suspension was transferred to a universal tube and the cells pelleted by centrifugation at 500 *x g* for 5 minutes. The supernatant was discarded and the cells resuspended in 10ml fresh medium ready to be seeded into flasks. Cells were passaged when confluent (approximately every 3-4 days).

S11, NSO, EL4 and A20 cell lines were grown in T75 flasks (Nunc) seeded at 5 x 10^6 cells with supplemented RPMI 1640 (Gibco BRL) containing 10% FCS, 70μg/ml penicillin, 10μg/ml streptomycin, 50μM β-mercapto-ethanol (ME) (Sigma) and 2mM L-glutamine. To passage non-/semi-adherent cells, flasks were banged to dislodge cells, the total medium transferred to a universal tube and the cells pelleted by centrifugation at 500 *x g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in fresh medium ready to be seeded into new flasks. All cell lines were incubated at 37°C in equilibrium with humidified 5% CO_2.

2.5.7 Mammalian Cell Quantification

In order to quantify the number of viable mammalian cells in a suspension, a 1: 1 dilution was prepared with 0.1% Trypan blue (0.1% trypan blue (Gurr) in PBS). Cells were counted using a haemacytometer, unstained viable cells differentiated from blue stained dead cells.

2.5.8 Storage & Retrieval of Cells

For long-term storage of cell lines, cells were resuspended at 10^6 cells/ml in freeze down solution (10% dimethyl sulfoxide (DMSO) in foetal calf serum). Aliquots of 1ml cell suspension/cryovial were frozen gradually in a –70 °C freezer overnight. Cryovials were then transferred to liquid nitrogen tanks, -180°C, for indefinite storage.

Cells retrieved from liquid nitrogen storage were thawed rapidly by placing the cryovial in a 37°C water bath. The thawed cell suspension was transferred to a universal tube and 9ml of media was added slowly, with constant swirling. Cells were pelleted by centrifugation at 450 *x g* for 5 minutes, resuspended in 7ml fresh media and transferred into a T25 flask (Nunc).

2.5.9 Transfection of Mammalian Cells with DNA by Electroporation

For transfection, cells were harvested from monolayers of ~80% confluency. Cells, $2x10^6$, were resuspended in 800µl of their standard growth medium and added to an electroporation cuvette (Equibio). As a standard, 30 µg DNA of chosen plasmid for transfection was added to the cells and mixed by gentle pipetting. For co-transfection with viral DNA, 4µg of viral DNA and 30µg of chosen plasmid were added to the cells together. Using an Equibio electroporator the cells were electroporated (260V pulse for approximately 18msec, capacity 1050µF) and then resuspended immediately in 5ml medium. The 5ml was then split between two wells of a six well plate (Nunc), each well made up to 5ml with medium and incubated at 37°C in equilibrium with humidified 5% CO_2. The medium was changed on the wells 24 hours later and selective agents applied if required.

2.6.1 Ecdysone-Inducible Mammalian Expression System

Following transfection of mammalian cells with the ecdysone inducible expression vectors, by electroporation, cells in 6 well plates were incubated for 24 hours. The medium was then replaced with fresh medium containing the expression induction agent, Ponasterone A (Invitrogen) at a final concentration of 5µM. Cells were assayed for gene expression 24 hours post induction.

2.6.2 Generation of Stable Mammalian Cell lines

In order to generate a stable cell line expressing pVgRXR, 48 hours post transfection with plasmid, cells were split and seeded at 25% confluency into fresh medium containing Zeocin at 150µg/ml. In the same way, cells transfected with both pVgRXR and an ecdysone system expression plasmid were split into medium containing 150µg Zeocin and 150µg hygromycin. Cells were fed with selective

medium every 3-4 days until foci could be identified. The antibiotic resistant cell foci were ring cloned. Ring cloning involved removing the medium from the well and placing a sterile ring (cut from the base of a gilson pipette tip) with a coating of silica gel (to form a seal) on top of a focus of cells. The cells were harvested by a versene wash followed by trypsinisation. Cells were transferred to a well of a 24 well plate and grown up in selective medium.

Generation of stable cell lines containing pBabe/puro was also performed by ring cloning. The selection media used contained puromycin at 2μg/ml. Cells were selected and subsequently maintained under selection continuously.

2.6.3 β-galactosidase Staining

To identify cellular expression of β-galactosidase, cell monolayers were fixed with 0.5% glutaraldehyde in PBS for 15 minutes. Following two short washes in PBS the cells were washed twice in detergent solution (40ml PBS, 1mM $MgCl_2$, 0.02% Nonidet P40 (Fisons), 0.01% Na deoxycholate) for five minutes. The cells were then covered in X-gal solution (20ml detergent solution plus 2ml Fe/Fe solution (50mM potassium ferricyanide and 50mM potassium ferrocyanide), 100μl X-gal stock (40mg/ml X-gal (Sigma) in DMSO)). Plates were incubated at 37°C on a shaker for 1-5 hours to allow β-galactosidase positive cells to stain blue. Wells were rinsed with water and air dried.

2.6.4 Immunofluorescence Staining

To prepare slides of *in vitro* cultured mammalian cells for immunofluorescence staining, 5μl of cells suspended at 10^8 cells/ml in PBS was applied to a glass slide and allowed to air dry on. Slides were then dipped in acetone for 5 minutes to fix the cells. The slides were allowed to dry and stored at 4°C if not used immediately. To block the slides 100μl of 5% normal pig serum in PBS was applied to the cells and slides were incubated at 37°C in a moist box (as with all further incubations) for 45 minutes. Slides were washed in PBS and then the primary antibody, diluted in PBS + 1% BSA (antibody dilutions used are displayed in section 2.5.4) was applied to the cells. After 2 hours incubation the slides were washed 3 times by immersing in PBS

for 5 minutes on a shaker each time. The secondary FITC conjugated antibody, diluted in PBS + 1% BSA, was applied to the cells and incubation was done for 1 hour. The slides were washed 5 times as before. The slides were mounted by dispensing a drop of 'Citifluor' (Citifluor Ltd. UK) onto the cells and placing a glass coverslip on top. Fluorescent stained cells were viewed using a UV light microscope at a wavelength of 450nm.

2.6.5 Radiolabelling Cells and Virions with [^{35}S] Methionine

Monolayers of C127 cells, approximately 80% confluent, in six well plates were infected with MHV-68 at a MOI of 5 pfu/cell ($5x10^6$pfu/10^6cells/well) or left uninfected and incubated at 37°C, 5% CO_2. After 24 hours the media was removed from infected and control wells and replaced with 5ml methionine free medium (Gibco BRL) plus 2mM L-glutamine. Plates were incubated for 30 minutes at 37°C with 5% CO_2. The methionine free media was then removed and replaced with 2ml labelling media (methionine free media plus 2mM L-glutamine with 1mCi/ml ^{35}S-methionine (Amersham) added). The plate was incubated for 4 hours at 37°C with 5% CO_2 in the presence of activated charcoal. The labelling media was then removed and the monolayer washed with PBS. Subsequently, the monolayer was scraped using a 1ml syringe plunger into 2 ml RIPA buffer (50mM Tris pH8.0, 150mM NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% SDS) or low ionic strength buffer (50mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 0.5% NP-40). If not used immediately, radiolabelled cell samples were stored at –20°C.

2.6.6 Radioimmunoprecipitation

Radiolabelled cell samples were dispensed into 500µl aliquots in screw capped microfuge tubes. Samples were precleared of proteins that bind to protein-A-sepharose (PAS) by the addition of 35µl 50:50 PAS (50:50 protein-A-sepharose was prepared by suspending 500 mg of dry PAS resin in 10ml TBS + 5% BSA +0.01% azide in a 15ml falcon tube, this was incubated at 4°C overnight allowing the PAS to hydrate. The PAS was washed three times by resuspending the resin in the TBS buffer and gentle centrifugation (500 *x g*). Finally the beads were resuspended at 50:50 in TBS buffer (2ml resin to 2ml buffer). This stock was stored at 4°C.

Following a 30 minute incubation at room temperature the resin was pelleted by centrifugation, 20 seconds at 2500 *x g*, and the supernatant was transferred to a new tube. Antibody; 2.5µl serum or monoclonal antibody, was then added to the supernatant. The tube was rotated for 2.5 hours at RT. PAS (50:50), 35µl, was then added to each tube and the incubation was continued for 30 minutes for precipitation to occur. The tubes were centrifuged briefly (20 seconds, 2500 *x g*) and the supernatant aspirated. The pellet was resuspended in 1ml wash buffer (the buffer the samples were originally harvested in i.e. RIPA buffer) and then repelleted by centrifugation as before. This process was repeated five times, before the final wash and the resuspended resin was transferred to a new tube to reduce background caused by radiolabelled protein adhered to the walls of the microfuge tubes. Following the final wash the resin pellet was resuspended in 35µl 1 x SDS-PAGE sample buffer. Tubes were spun to pellet the resin before removing samples of the supernatant for loading on SDS-PAGE gels.

2.6.7 Immunostaining on Paraffin Embedded Tissues

To dewax, paraffin sections cut onto biobonded slides were placed in xylene for 15 minutes followed by immersion in 100% ethanol for 5 minutes. The slides were then placed in 0.3% H_2O_2 in methanol (freshly made up) for 30 minutes. To hydrate the sections, slides were immersed in a series of ethanol dilutions; 100%, 95%, 70%, 50% and 30% for 30 seconds each and then two times in H_2O for one minute. Followed by immersion of slides in 1% Triton X-100 in H_2O for 90 seconds and then two times in H_2O for 5 minutes. Sections were digested with 10mg/ml proteinase K in 20mM Tris, 2mM $CaCl_2$, pH 7.0 for 20 minutes at 37°C. Next, slides were incubated in PBS plus 2g/L glycine and 5mM EDTA for 5 minutes and then PBS for 3 minutes. To block, prior to immunostaining, slides were placed flat in a moist chamber, 2% normal goat serum (NGS) in PBS was dispensed onto each section and incubation was done for 30 minutes - overnight at room temperature. Primary antibody, diluted (dilutions used for antibodies are shown in section 2.5.4) in 2% NGS, was applied to slides. Following 2 hours, incubation slides were washed in PBS 3 times for 5 minutes each. Secondary antibody, biotinylated goat anti-rabbit IgG diluted 1/500 with 2% NGS in Vector Laboratories solution BA1000 was

applied to sections and incubated for 2 hours. Slides were washed 3 times as before. Vector Laboratories solution PK4000 was applied to the sections and slides were incubated for 30 minutes followed by 3 washes as before. Following addition of the DAB (diaminobenzidine) substrate (Sigma Fast DAB) sections were monitored closely for colour change. To stop exposure, slides were washed in PBS and then H_2O. Haematoxylin was used to counterstain the sections and then the slides were dehydrated through the ethanol dilutions (in reverse order to the hydration process) and mounted in DPX/xylene.

2.6.8 MHV-68

Murine gammaherpesvirus 68 (MHV-68) was originally isolated from *Clethrionomys glareolus*, a bank vole, in Slovakia (Blaskovic *et al.*, 1980). The G2.4 infectious clone used in these experiments was originally derived from MHV-68 stock supplied by Professor Blaskovic to the Department of Pathology, Cambridge University.

Working stocks of MHV-68 were prepared by infection of BHK-21 cells, in suspension, at a multiplicity of infection (MOI) of 0.01. Suspensions were incubated for 1 hour on a shaker at 37°C. Following infection cells were transferred to T175 Nunc tissue culture flasks containing 50 ml ETC medium and incubated for 6 to 7 days at 37°C, 5% humidified CO_2. Monolayers were dislodged by tapping the flasks and using a scraper (Sterilin), the cells were pelleted by centrifugation at 2000 *x g* for 30 minutes at 4°C. The supernatant was discarded and cells were transferred to a Dounce homogeniser (Wheaton, USA) which was used to disrupt the BHK cells. The homogenised suspension was centrifuged at 2000 *x g* for 30 minutes at 4°C. The supernatant (containing virus) was stored on ice whilst the pellet was homogenised a second time and recentrifuged at 2000 *x g* for 30 minutes at 4°C. Supernatants were pooled and dispensed into 200µl or 500µl aliquots and stored at –70°C until use. BHK lysate was prepared in the same way omitting infection of cells.

2.6.9 Vaccinia Virus

Vaccinia virus recombinants, VV^{gp150} and VV^{gpt}, had been generated previously by Dr. J P Stewart using vaccinia strain WR (Stewart *et al.*, 1999). Vaccinia virus

working stocks were prepared by infection of CV-1 cell monolayers in T175 flasks at a MOI of 0.01. After 3 days incubation at 37°C, 5% humidified CO_2, virus was harvested by the same method used for MHV-68 virus preparation.

2.7.1 Inbred Mice Strains

For *in vivo* work the mouse strains used were supplied by Bantin & Kingman aged 3 – 4 weeks of age on arrival and included:

BALB/c Haplotype H-2^d

C57BL/6 Haplotype H-2^b

2.7.2 Infection of Experimental Animals

Prior to intranasal infection with virus, mice were anaesthetised using halothane (BOC Gases). For infection with MHV-68 and vaccinia virus recombinants, the required titre of virus was diluted in 40µl and 50µl PBS, respectively, and administered to the nose of the animal. To remove organs for analysis, animals were killed by cervical dislocation and the lungs and spleen immediately dissected from the cadaver. Lungs were placed in cryovials and frozen at –70°C, spleens were deposited into 5 ml medium and stored on ice for infectious centre assays on the same day.

2.7.3 Virus Titration

Organs to be assayed for MHV-68 titre were stored at –70°C, thawed rapidly at 37°C and kept on ice. The organ was homogenised in a glass homogeniser (Soham Scientific, UK) with 1ml cold BHK culture media. The homogenate was transferred to a cryovial and frozen at –70°C. The homogenate was thawed rapidly as before and centrifuged at 2000 *x g* for 5 minutes at 4°C to pellet cell debris.

Tissue homogenates or virus samples were diluted in bijoux, in duplicate, in a series of 10 fold dilutions. To each bijoux a 200µl suspension of 10^6 BHK cells were added. The dilutions, at a final volume of 2ml of BHK growth medium were incubated at 37°C on a shaker (200 rpm) for 1 hour to allow virus adsorption. Following absorption samples were mixed by vortexing and then each sample was

poured into a 60mm petri dish (Falcon) containing 4ml medium. Petri dishes were gently swirled to evenly distribute cells and incubated at 37°C, 5% CO_2, for 4 days. The medium was removed and 3ml of 4% formal saline was added to each dish to fix the cell monolayers. Following fixation for a minimum of 15 minutes the formal saline was removed and 0.1% toludine blue was added to stain the cells. After a minimum of 15 minutes the toludine blue was poured off and the plates rinsed gently in water and allowed to dry. Virus plaques were visualised and counted using a light microscope. Virus titre was referred to as plaque forming units (pfu).

For titration of vaccinia virus, organs were prepared in the same way as for MHV-68 however, serial dilutions were applied to confluent monolayers of CV-1 cells in six well plates. Following a 1 hour incubation at 37°C, 5% CO_2, to allow for virus adsorption the inoculum was replaced with 5ml medium. Cells were fixed and stained following 3 days of incubation at 37°C, 5% CO_2.

2.7.4 Infectious Centre Assay

To detect infectious centres, the spleen of infected animals was removed immediately after death and placed into 5ml of ice cold supplemented RPMI (as in section 2.5.6). The cells were teased from the spleen using sterile forceps in a 60mm petri dish containing 5ml supplemented RPMI. The cells were transferred to a universal tube and pelleted by centrifugation at 500 *x g*, 4°C for 5 minutes. The supernatant was discarded and the cells resuspended by gently tapping the universal tube. In order to lyse the red blood cells, 1ml of sterile H_2O was added to the cells and then immediately afterwards 9ml of PBS was added. The cells were pelleted as before and resuspended in 10ml supplemented RPMI. Large clumps of red cell ghosts were allowed to settle and then the cell suspension was carefully pipetted off, transferred to a bijoux and kept on ice. Petri dishes were set up with 6ml supplemented RPMI containing 10^6 fresh BHK cells in suspension. Spleen cells were counted and then, in duplicate, a 10 fold series of cell numbers were added to the 60 mm Petri dishes e.g. 10^7, 10^6 and 10^5 splenocytes/petri dish. Remaining splenocytes, not plated out, were frozen at –70°C to lyse the cells, for subsequent infectious virus titration. The plates were incubated for 5 days at 37°C, 5% CO_2.

Plates were fixed and stained as described for plaque assays. Numbers of plaques/ plate were counted using a light microscope and the infectious centre titre was calculated per 10^7 splenocytes and per spleen.

2.7.5 Plaque Purification

Cells harbouring virus that had under gone homologous recombination with the recombinant virus plasmid constructs formed plaques expressing the green fluorescent protein (GFP). These were detected by fluorescence microscopy using a UV filter (excitation at 450 ± 25nm). Plaques expressing GFP were picked, using a pasteur pipette, from cell monolayers that had been overlayed with agar (2% low melting point 'Seaplaque' agar (Flowgen) in PBS mixed with an equal volume of media) 24 hours post transfection, and each transferred to 1ml ETC media in a glass universal. The universals were sonicated for 60 seconds in a bath sonicator. The picked plaque suspensions were serially diluted from 10^{-1} to 10^{-5} and 400µl of each dilution was inoculated onto BHK cell monolayers in six well plates. After a 1 hour incubation at 37°C to allow adsorption, the inoculum was removed and cell monolayers were washed with 5ml of ETC media. An agar overlay, as above, was applied to each well. Four days after infection green plaques were picked, serially diluted and plated out as before. This process was repeated at least four times in duplicates.

As an alternative to plaque picking, virus cloning was also done by inoculating a series of 2 fold dilutions of virus samples onto BHK monolayers in 96 well plates. Cells of wells containing one virus plaque, expressing GFP, were scraped into a volume of 200µl of cell culture media and each transferred to a glass universal. The universals were bath sonicated for 60 seconds to lyse the cells. The suspensions were then used for subsequent dilutions and inoculation into BHK monolayers in 96 well plates. The same process was used when C127 cells were used instead of BHK cells.

2.7.6 Virus Neutralisation Assay

To assay the ability of antisera to block MHV-68 infection a standard plaque reduction assay was done. Sera were heat treated at 56°C for 30 minutes to destroy complement. Virus, 100pfu, was incubated with the heat treated antisera diluted in phosphate buffered saline at a final volume of 50µl. After an hour incubation at 37°C the virus/antiserum mix was added to confluent monolayers of C127 cells in 6 well plates in a final volume of 1ml standard media. Following incubation at 37°C for one hour the virus/antiserum mix was removed, the monolayer washed with medium and then 6 ml of medium added. Plates were incubated for 5 days at 37°C, 5% CO_2 and then fixed with 4% formal saline, stained and counted as for a standard plaque assay.

2.7.7 Analysis of Protein Blocking MHV-68 Infection

Confluent C127 cell monolayers in six well plates were incubated with fusion protein, 2µM in 500µl standard C127 culture media for one hour at 4°C on a shaker. Virus, 200 pfu was added in 200µl media and incubation was continued for one hour at 4°C and then shifted to 37°C for one hour to allow penetration of virus into cells. The monolayer was washed twice with an excess of low pH buffer (40mM citric acid, 10mM KCL, 135mM NaCl, pH3.0) to inactivate and remove remaining extracellular virus (Boyle & Compton, 1998). The standard C127 culture media, 6ml, was added to each well. Plates were incubated for 5 days at 37°C, 5% CO_2 and then fixed with 4% formal saline, stained and counted as for a standard plaque assay.

In addition, the ability of fusion protein to block infection in suspension was done in oredr to reduce the volumes used, 10^6 cells were suspended in 250µl media containing 2µM fusion protein in a 1.5ml microfuge tube. Tubes were rotated at 4°C for one hour. Virus, 200 pfu in 100µl of media was added and tubes were incubated for one hour at 4°C. The temperature was shifted to 37°C for one hour. Cells were pelleted by centrifugation at 500 *x g* and washed twice with 1ml of low pH buffer as above, pelleted and resuspended in C127 culture media. The cell suspension was transferred to a well of a six well plate made up to 6ml with culture media.

2.7.8 Preparation of Serum from Blood

After animals were killed by cervical dislocation blood was collected from the heart. To prepare serum blood was incubated overnight at 4°C in 0.2ml microfuge tubes to allow clotting to occur. The following day tubes were centrifuged at 10,000 *x g* for 20 minutes. The upper layer of serum was transferred to a new tube and stored at -20°C.

2.7.9 MHV-68 Enzyme-Linked Immunosorbent Assay (ELISA)

Reagents:

Borate buffered saline (BBS): Boric acid, 61.85g, disodium tetraborate 95.4g, NaCl 43.85g made up to a final volume of 10 litres in distilled water, pH 8.2.

Blocking buffer: BBS plus 2% normal rabbit serum.

Wash buffer: BBS plus 0.05% (v/v) Tween 20.

Antigen dilution buffer: BBS plus 1% normal rabbit serum, 0.01% Tween 20.

Carbonate/bicarbonate buffer (pH 9.8): - 22ml of 0.2M sodium carbonate solution added to 28ml 0.2M sodium bicarbonate solution.

UV inactivated MHV-68: MHV-68 stock ($3.8x10^8$ pfu/ml) was UV irradiated for 30 minutes.

HRP substrate solution: Sigma Fast™ OPD peroxidase substrate tablet set – one *o*-phenylenediamine dihydrochloride (OPD) tablet and one urea hydrogen peroxidase/buffer tablet dissolved in 20ml H_20 – yields a solution of 0.4mg/ml OPD, 0.4mg/ml urea hydrogen peroxide and 0.05M citrate phosphate buffer.

The mouse MHV-68 sera conversion ELISA was carried out (in accordance with Usherwood *et al*, 1996a) on mouse sera in order to determine the level of MHV-68 specific antibody. In preparation, wells of Immulon 4 96 well plates (Dynatech), were coated with rabbit anti-MHV-68 hyper-immune serum diluted 1/1000 in carbonate/bicarbonate buffer, 100µl was added per well and the plate incubated overnight at 4°C. The plate was flicked to remove the serum solution and then blocked by the addition of 100µl/well of blocking buffer and incubation at 37°C for

one hour. The plate was washed six times in wash buffer and then tap water each time. After the final wash 100µl of UV inactivated MHV-68 antigen diluted 1/64 in antigen dilution buffer was added per well and the plate incubated for one hour at 37°C. The plates were washed six times as before. Each mouse serum sample was diluted 1/20 and titrated in 1/2 serial dilutions down the plate. As a positive control MHV-68 hyperimmune mouse serum diluted at 1/400 and as a negative control normal mouse serum diluted at 1/20 were titrated down the plate in 1/2 serial dilutions. The total volume per well at this stage was 100µl. The plate was incubated for one hour a 37°C and then washed as before. Horseradish peroxidase (HPX) conjugated rabbit anti-mouse immunoglobulin was diluted to 1/2000 in antigen dilution buffer and 100µl added per well. Alternatively, at this stage HPX conjugated rabbit antisera against different mouse immunoglobulin isotypes was used (refer to antibody dilutions in section 2.5.4). Following incubation for 1 hour at 37°C the plates were washed 6 times as before. 100µl of HRP substrate solution was then added to each well. After 15 minutes incubation at room temperature the reaction was stopped with 100µl of 12.5% (V/V) sulphuric acid. The sample OD value was measured at 490nm using an ELISA plate reader (DYNATECH MR5000). To determine the relative levels of MHV-68 specific antibody OD values were plotted on a standard curve generated from the positive control MHV-68 hyperimmune mouse serum values, calculated as 1/mean OD values per dilution.

2.8.1 Preparation of Splenic Lymphocytes for Binding Assays

For preparation of splenocytes for FACS binding assays, a fresh spleen was removed from a Balb/c mouse and placed into a 60 mm petri dish containing 5 ml of chilled complete RPMI medium. Using sterile forceps the spleen cells were teased out into the medium. Spleen casing and clumps were discarded, the contents of the petri dish was pipetted into a universal. The cells were pelleted by centrifugation at 500 *x g*, 4°C for 5 minutes. Cells were resuspended in 10ml medium and the clumps allowed to settle. The medium was then transferred to a universal tube containing 10ml of a lymphoprep reagent; histopaque (Sigma). Pipetting was done slowly to ensure the media formed a separate layer on top of the histopaque. Following centrifugation at 2000 *x g* for 20 minutes, brake off, the layer of lymphocytes that formed between the

media and the histopaque was gently pipetted off using a pasteur pipette. The lymphocytes were transferred to a fresh universal and the volume made up to 20ml by the addition of ~15 ml media. The cells were centrifuged at 900 *x g* for 10 minutes, brake off, resuspended in 20ml fresh media and centrifuged a further 10 minutes at 500 *x g*, brake off. The cells were resuspended in 20ml fresh media, a sample was taken for counting and the cells pelleted as before and resuspended in media at the required cell concentration.

2.8.2 MACS Preparation of B Cells

B cells were purified from splenic lymphocyte preparations for binding assays by magnetic associated cell sorting (MACS). Splenic lymphocytes were resuspended in ice cold 1% foetal calf serum in PBS (PBS/FCS) at a concentration of 10^8 cells/900µl. To 900µl of cell suspension 100µl of anti-CD43 microbeads (Miltenyi Biotec GmbH) was added. The mixture was incubated at 4°C for 15 minutes and then made up to 15 ml with FCS/PBS. The cells and beads were pelleted by 5 minutes centrifugation at 500 *x g* and then resuspended in 1ml PBS/FCS, this was then loaded onto a MACS column (type BS, capacity 10^8 cells, Miltenyi Biotech GmbH) held in the magnetic cell sorter apparatus (Miltenyi Biotech GmbH). The column was washed with 15 ml PBS/FCS and the total flow through (containing the purified B cells) was collected. The cells were pelleted as before and resuspended in medium as quickly as possible. To assess the purity of the B cells FACS staining was done.

2.8.3 FACS Analysis & Binding Assay

Cells, 10^6 per well of a 96 round bottom well plate (Nunc), were suspended in 200µl FACS buffer (PBS, 1% bovine serum albumin (BSA), 0.2% sodium azide) for 45 minutes. Cells were pelleted by centrifugation at 500 *x g*, brake off, for 5 minutes and resuspended in either a total of 50µl of fusion protein preparation, 50 µl of MHV-68 virus preparation or 50µl of BHK cell lysate each diluted in FACS buffer with 0.2% sodium azide present and incubated at 37°C for 1.5 hours. Cells were washed 3 times in FACS buffer. The washing process consisted of pelleting the cells by centrifugation at 500 *x g*, (brake off) for 5 minutes, pipetting off the supernatant

gently by hand, resuspending the cells by vortexing the plate and then adding FACS buffer. Cells were resuspended in 50µl primary antibody, either rabbit anti-gp150 antibody or anti-His antibody diluted (1:250 and 1:50 respectively) in FACS buffer and incubated at 4°C for 60 minutes. Cells were washed as before and then incubated with the secondary swine anti-rabbit FITC antibody (30µl/well) for 30 minutes at 4°C. Following this, cells were washed 5 times in FACS buffer and then resuspended in 200µl of 1% formal saline. Cells were sorted using a FACStar (Becton Dickinson) FACS machine operated by Alan Ross, a technician within the department. Routinely events from 10,000 cells were used for analysis. The marker for positive cells was postioned at 1% of the events from primary and secondary antibody alone. When splenocytes were used lymphocytes were discriminated for by cell size and granularity.

FACS staining for cell surface markers was done as the above method (omitting the initial protein incubation step), the anti cell surface marker antibody as the primary antibody followed by the appropriate anti-primary Ig species secondary antibody if required (working dilutions of antibodies in FACS buffer are shown in section 2.5.4). If double staining was done the primary antibodies were added simultaneously.

2.8.4 ELISA Binding Assay

C127 cells were seeded at 1 x 10^4cells/well of a flat bottomed 96 well plate (Nunc). After 12 hours, wells contained confluent monolayers required for the cell binding assay. Cells were washed with PBS and then fixed with 4% paraformaldehye in PBS for 45 minutes. Wells were washed twice with PBS and then 100µl of normal pig serum diluted at 1:500 in FACS buffer was added to wells as a block. Following 30 minutes incubation at RT the block was removed and either 50µl fusion protein preparation, 50µl MHV-68 virus preparation or BHK lysate, diluted in FACS buffer, was added to wells and incubated for 1.5 hours at 37°C. Wells were washed 3 times with PBS and then 50µl of primary antibody (anti-gp150 antibody or anti-His antibody) was added. After 1 hour at RT wells were washed 3 times with FACS buffer. Alkaline phosphatase conjugated secondary antibody was added, 50µl, and wells were incubated for 30 minutes at RT. The plate was then washed 5 times with

PBS and 50µl/well of alkaline phosphatase substrate NBT/BCIP (Sigma) was added. After a 60 minute incubation at RT the optical density of the wells was read, at 405nm wavelength, using a Dynatech MR5000 ELISA plate reader.

Gene Gun Reagents & Methods

2.8.5 Precipitation of DNA onto Microcarriers

To 49mg of 1.6µm gold microcarriers (Biorad) in a 1.5 ml microfuge tube 120µl of 0.1M spermidine (Sigma) was added. The tube was vortexed for a few seconds and then sonicated for 5 seconds to break up clumps of gold particles. To the gold/spermidine mix 100µl of 1mg/ml DNA was added. The tube was vortexed for <5 seconds. While vortexing the tube at a moderate rate, 120µl of 1M CaCl was added dropwise to the mixture. The mixture was allowed to precipitate at RT for 10 minutes. The tube was spun briefly at 2000 *x g* to pellet the gold. The supernatant was discarded and the pellet resuspended by flicking the tube gently. The pellet was washed 3 times with 1ml 100% ethanol, between each wash the gold was pelleted by a brief spin at 2000 *x g*. After the final ethanol wash the pellet was resuspended in 200µl of 100% ethanol containing 0.01mg/ml polyvinylpyrrolidone (PVP) (Biorad). The mixture was transferred to a 15ml polypropylene Falcon tube and the volume made up to 7ml with the ethanol /PVP solution. If not used immediately for tube preparation the DNA/microcarrier suspension could be stored at -20°C for up to two months.

2.8.6 Loading the DNA/Microcarrier Suspension into Gold-Coat Tubing

A 75cm length of Gold-Coat tubing (Biorad) was loaded into a Tubing Prep Station (Biorad) and high grade nitrogen gas (99.998%) was allowed to flow through the tubing at 0.3-0.4 litres per minute (LPM) for at least 15 minutes prior to use to ensure the tubing was dry. The tubing was attached to a peristaltic pump and then approximately 3 ml of microcarrier/DNA suspension, while being gently vortexed, was drawn into the tubing. The tubing was quickly brought to a horizontal position and slid into the Tubing Prep Station. During this procedure the microcarriers settled

in the tube and the ethanol was immediately pumped from the tube. The tube was then rotated 180° to allow the gold to begin coating the inside of the tubing. After 5 minutes the tubing was rotated continuously. Following the first 5 minutes of rotation the nitrogen gas supply was turned on at 3.5 LPM to dry the Gold-Coat tubing while rotating for a further 15 minutes. The tubing was removed from the Tubing Prep Station and cut into 1.27cm bullets. Each bullet was examined and those insufficiently coated with gold were discarded. The bullets were stored with desiccant in a tube sealed with parafilm at 4°C.

To quantitate the DNA in a bullet the inside of the bullet was flushed with 500µl H_2O to elute the gold particles. The suspension was centrifuged briefly at 600 *x g* to pellet the gold particle and the amount of DNA present in the supernatant was quantified by fluorometry as described in section 2.1.3. Routinely, approximately 1µg of DNA was present per bullet.

2.8.7 *In Vitro* Particle Delivery

To test the expression of plasmid from gene gun bullets *in vitro*, C127 cells at 80% confluency in six well plates were used. The cells were washed once with DME containing no additives. The wash was removed. The bullet for testing was loaded into the Helios Gene Gun (Biorad) and the cells were fired on directly at a pressure of 40 psi of high grade (99.995%) helium (BOC Gases). Standard growth medium for C127 cells was replaced in the wells immediately post gene gun delivery. Cells were harvested 6 and 24 hours post firing to assay for mRNA expression (refer to section 2.3.5).

2.8.8 *In Vivo* Particle Delivery

Prior to delivery of DNA into the abdominal epidermis, the abdominal fur of each mouse was clipped as closely as possible using an electrical minishaver (Wahl). The spacer on the nozzle of the Helios Gene Gun was held against the abdomen of the mouse. The DNA coated gold particles were discharged into the skin at a pressure of 500 psi. One bullet was discharged per animal per immunisation with each plasmid. Gene gun mediated immunisations were carried out without the use of anaesthesia.

For tissue sections, the target animal was killed by cervical dislocation and the area of the skin which was fired onto was excised using scissors. For fixation, the skin sample was placed into 10% formal saline overnight and then removed to 70% ethanol. Samples were embedded in paraffin wax and 6μm sections were cut onto biobonded slides. Haematoxylin and eosin (H & E) staining was performed by services within the Veterinary Pathology Department.

RESULTS

3.1.1 RECOMBINANT VIRUS

Genetic studies of herpesviruses have provided a considerable amount of information on the biology and molecular biology of these viruses. An important approach used to determine the function and importance of viral genes for the replication of herpesviruses is the study of mutant and recombinant viruses in which specific genes are dysfunctional or mutated. Mutant viruses have been isolated from herpesvirus populations on the basis of altered plaque phenotype (Ejercito *et al.*, 1968), drug resistance (Kit & Dubbs, 1963, Tognon *et al.*, 1988, Pelosi *et al.*, 1998) and host range properties (Aurelian & Roizman, 1964) . With the power of molecular cloning techniques it is possible to generate recombinant viruses with specific genes of interest made dysfunctional by insertion into, mutation and deletion of the gene of interest. With regard to herpesvirus glycoproteins many mutant viruses, particularly for HSV-1, have been generated and isolated with dysfunctional glycoprotein genes (e.g. Forrester *et al.*, 1992, Balan *et al.*, 1994, Dijkstra *et al.*, 1996, Wang & Hutt-Fletcher, 1998). This has provided the opportunity to determine the function of each individual glycoprotein in the context of the virus lifecycle by studying the phenotype of the recombinant viruses in comparison with that of wild type virus *in vitro* and *in vivo*.

The inability of EBV and KSHV to grow permissively in cell culture systems has hampered the study of the molecular biology of these gammaherpesviruses. In contrast the relatively efficient replication of MHV-68 *in vitro* indicates potential for more amenable genetic manipulation to generate recombinant viruses. In order to determine the function of gp150 the aim was to generate a gp150 'knock out' (KO) virus, unable to express gp150.

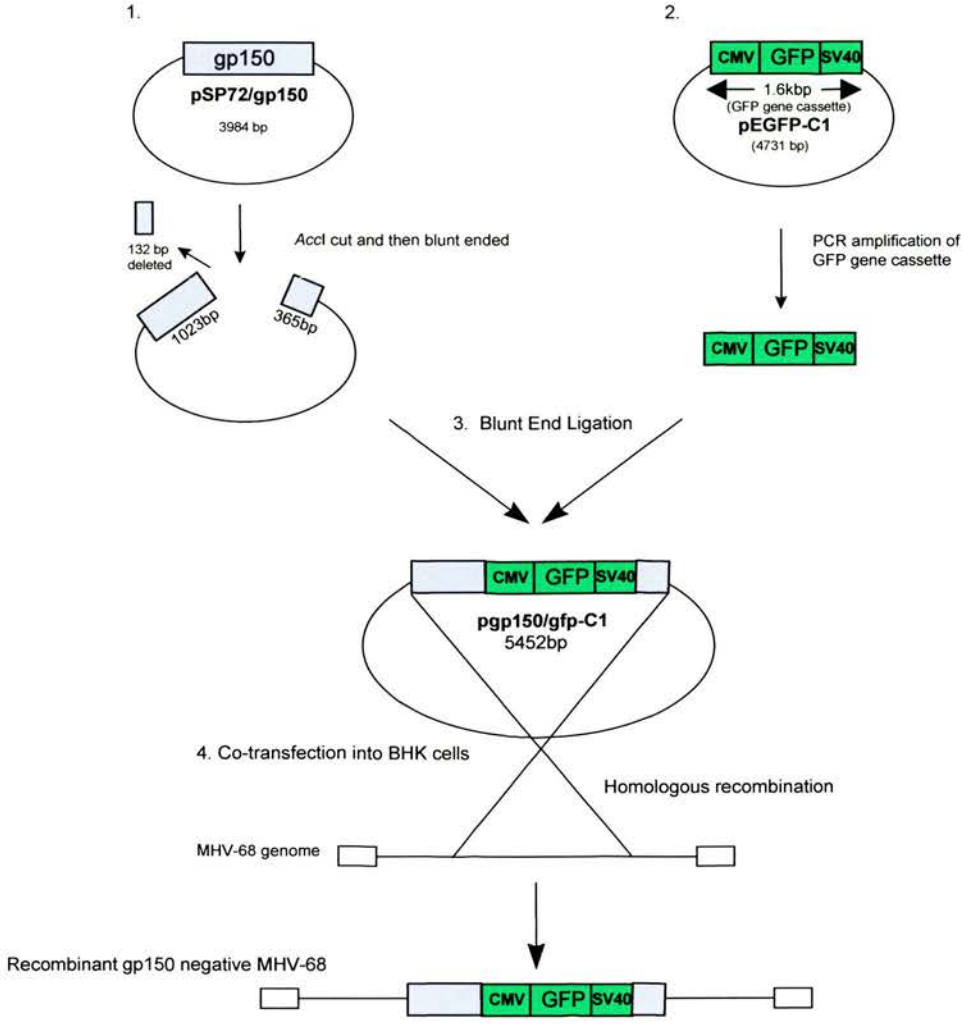
3.1.2 Gp150 KO Recombinant Virus – Cloning Strategy

The preliminary strategy used to generate a gp150 negative recombinant virus is summarised in figure 3.1.1. The intention was to delete part of the gp150 gene and insert a gene cassette encoding green fluorescent protein (GFP) into the gp150 gene within the virus genome using homologous recombination. GFP, originally derived

from the jelly fish *Aequoria victoria*, fluoresces green upon excitation by UV light (450nm wavelength) (Zolotukhin *et al.*, 1996). Within the gene cassette the GFP gene is under the control of the constitutive CMV immediate early promoter (CMVIE) and GFP would therefore be expressed during productive replication of the virus and hence act as a fluorescent marker for the selection of recombinant viruses. This marker is particularly advantageous as it allows early detection of recombinants before extensive cytopathic effect is observed and visualisation of GFP by UV microscopy avoids disruption of the continued growth of the virus. GFP is a tool increasingly used in molecular biology detection systems including the identification of recombinant herpesviruses (Duboise *et al.*, 1996, Delecluse *et al.*, 1998, Prichard *et al.*, 1999).

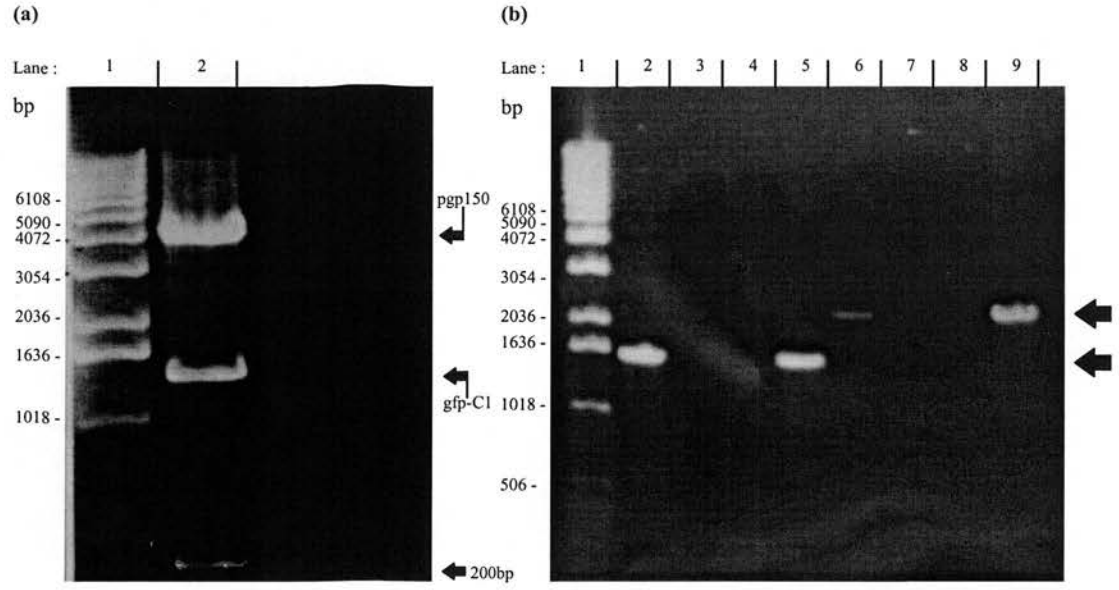
The plasmid pSP72/gp150 encoding the entire gene for gp150 was digested with restriction enzyme *Acc*I. This digest resulted in the removal of a 132bp fragment from the gp150 gene (for position of *Acc*I sites in *gp150* see figure 1.8). Following this the *Acc*I cut ends were blunt ended using T4 DNA polymerase. A 1.6kbp DNA fragment containing the GFP gene, flanked by the CMV immediate early promoter (CMVIE) and the Simian virus 40 polyadenylation signal (SV40pA) was amplified from the plasmid pEGFP-C1, by PCR using *Pfu* DNA polymerase and primers CMV1 and SV401 (refer to section 2.1.8 for primers and PCR conditions). The PCR product was purified and blunt end ligated into the prepared cut pSP72/gp150. The deletion of part of the gp150 gene was designed to ensure that in the event that the GFP cassette was lost from the recombinant virus genome the gp150 gene would remain dysfunctional and the virus would be unable to revert to wild type. The left and right hand gp150 sequence flanking the GFP cassette, required for homologous recombination with complementary sequences in the viral genome, were 1023bp and 365bp in length, respectively. The ligation mix was used to transform competent *E. coli* by the heat shock method. Of the 200 colonies that grew, selected using ampicillin, 12 were picked, grown up in liquid culture and their plasmid DNA extracted. One plasmid preparation was identified which contained the GFP cassette, this produced the correct banding pattern (bands of 4kbp, 1.4kbp and 200bp) following digestion of the plasmid preparations with *Acc*I as shown in figure 3.1.2.a.

Figure 3.1.1 Cloning strategy for generation of a gp150 negative recombinant virus



The method designed to generate a recombinant MHV-68 virus lacking a functional gp150 gene and encoding the green fluorescent protein (GFP) gene cassette. Step 1. pSP72/gp150 is cut with *Acc*I and blunt ended. Step 2. A 'GFP' gene cassette containing the GFP gene flanked by a CMV immediate early promoter (CMV) and the SV40 polyadenylation signal (SV40), is cloned by PCR using '*Pfu*' DNA polymerase. Step 3. The cloned gene cassette is blunt end ligated into the *Acc*I cut pSP72/gp150 to create pgp150/gfp-C1. Step 4. Purified MHV-68 genomic DNA and the pgp150/gfp-C1 are co-transfected into BHK cells by electroporation. Homologous recombination between the gp150 gene of MHV-68 and the disrupted gp150 gene of pgp150/gfp-C1 yield a recombinant gp150 knockout MHV-68.

Figure 3.1.2. Analysis of pgp150/gfp-C1 plasmid construct for the generation of a gp150 knock out recombinant virus

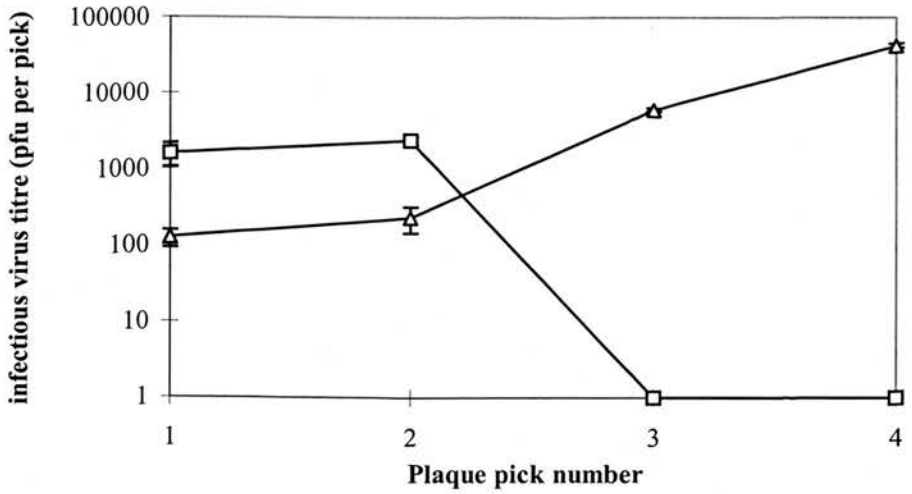


Restriction digestion and polymerase chain reaction (PCR) analysis of pgp150/gfp-C1 to confirm green fluorescent protein (GFP) encoding cassette insertion and orientation. (a) Restriction digest of pgp150/gfp-C1 using *Acc*I, Lane - 1: 1 kbp marker, 2: pgp150/gfp-C1, bands at ~4kbp, 1.4kbp and a faint band at 200bp. (b) PCR to determine presence and orientation of *gfp* in pgp150/gfp-C1. Primers for *gfp*; CMV1 & SV40 1 used to determine presence of *gfp* (lanes 2-5) primers PAG1 & SV40 1 were used to check orientation of *gfp* (lanes 6-9). Lane - 1: 1 kbp DNA ladder marker, 2: pEGFP-C1 positive control, 3 & 7: pSP72/gp150 negative control, 4 & 8: pSP72/gp150 negative control, from small scale DNA preparation, 5 & 9: pgp150/gfp-C1 small scale DNA preparation, 6: pgp150/gfp-C1 ligation mix positive control.

PCR was used to determine the orientation of the inserted gene cassette. PCR using the primers PAG1 (positioned in gp150) and SV401 produced a 2kbp product confirming that the GFP insert was orientated in the same transcriptional orientation as the gp150 gene it had been inserted into as shown by figure 3.1.2.b (refer to section 2.1.8 for primers and PCR conditions). A large scale preparation of this plasmid, called pgp150/gfp-C1, was made ready using the CsCl method, for transfection into mammalian cells. Pgp150/gfp-C1 (30μg) was transfected into BHK cells to check for expression of GFP, by UV microscopy green fluorescent cells could be identified 6 hours post transfection.

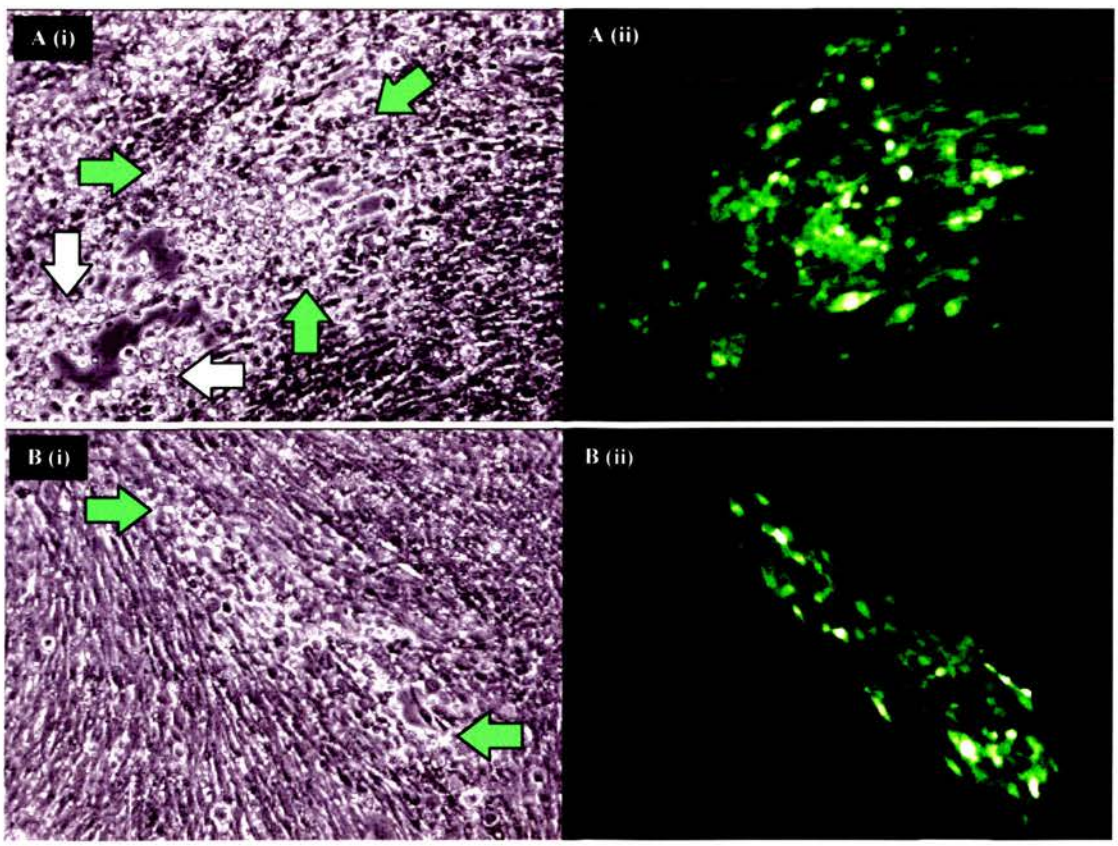
Pgp150/gpf-C1 (30μg) and purified MHV-68 genome (5μg) were co-transfected into BHK cells by electroporation. By 24 hours post transfection cells expressing GFP, exhibiting green fluorescence, were detected. The green fluorescent areas of cells spread within the monolayer and green fluorescent plaques had formed by four days post transfection. Present with the 'green' plaques were also 'white' wild type (non-fluorescent) plaques at an approximate ratio of 1:12. Green plaques were picked and the virus contained within was serially diluted and plated with fresh BHK cells which also gave rise to both 'green' and 'white' plaques at an approximate ratio of 1:9 respectively. This process was repeated a further two times by which point only 'green' plaques could be observed (see figures 3.1.3. & 3.1.4.). 'Green' plaques did not differ in morphology or development rate in comparison to wild type plaques.

Figure 3.1.3. Plaque purification of recombinant virus expressing green fluorescent protein



To generate a recombinant gp150 knock out virus MHV-68 DNA (5μg) and pgp150/gfp-C1 (30μg) were co-transfected into BHK cells. Plaque purification was done on BHK cells to purify virus expressing green fluorescent protein (GFP) from wild type virus. The levels of wild type MHV-68 (squares) and GFP expressing recombinant virus (triangles) present in sequential plaque picks is shown. The limits of detection for plaque picks 1-4 were 10, 10, 100 and 1000 pfu respectively. This was due to the increasing titres of GFP expressing virus. The titres of virus expressing GFP were determined by UV microscopic examination and wild type virus were determined by light microscopic examination, following formal saline fixation and toluine blue staining of monolayers. Bars denote standard deviation of duplicate samples. Pfu- plaque forming units.

Figure 3.1.4. Recombinant MHV-68 expression of green fluorescent protein



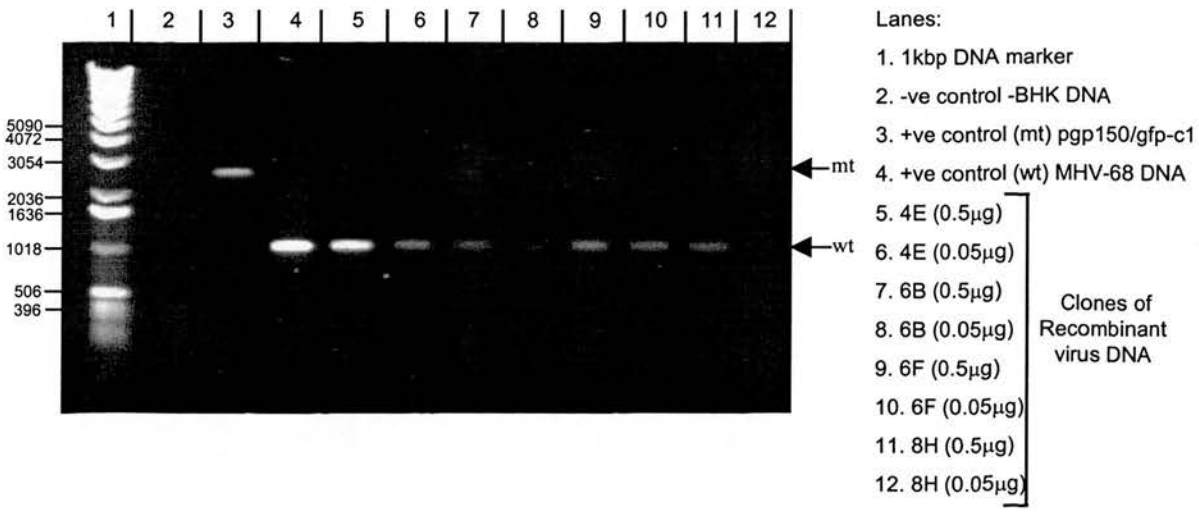
Plaques present in BHK cell monolayers three days after infection with virus derived from 'green' plaque picks of recombinant MHV-68 virus expressing green fluorescent protein (GFP), as viewed by phase contrast light microscopy (i) and by UV microscopy (ii). The GFP expressing plaques were produced in the generation of a gp150 knockout virus following the co-transfection of MHV-68 DNA and pgp150/gfp-C1 into BHK cells. Initial plaque picks (A) gave rise to both 'green' plaques (green arrows) and wild type 'white' plaques (white arrows). The later sequential plaque picks (B) gave rise to only gp150 KO 'green' plaques. Original magnification: 50x

3.1.3 DNA Analysis of 'Green' Virus

DNA analysis of the 'green' virus was performed to check that the virus was free from wild type MHV-68 and to determine if the desired homologous recombination with the gp150 gene had occurred. PCR was performed on DNA extracted from 'green' virus cultures to check for the presence of wild type gp150. At this stage these cultures had been passaged four times, DNA was extracted from limiting dilution cultures of the fifth passage. Primers PAG11 and MA2 were used which flank the region of gp150 into which the GFP cassette was inserted (for PCR conditions refer to section 2.1.8). The predicted product resulting from wild type was 1059 bp, the expected product generated from the recombinant virus construct was 2.68 kbp. As figure 3.1.5. shows, for all 'green' virus samples tested a wild type gp150 band was produced. The predicted recombinant virus band was detected for one clone (a faint band is present in lane 7 of figure 3.1.5.). The absence of recombinant gp150 bands in other samples, despite the visible presence of green fluorescent plaques, was likely to be due to competition from the considerably shorter wild type DNA target. This PCR cannot be used to quantify the ratio of wild type to recombinant gp150 present for this reason.

From this data it was clear wild type gp150 DNA was present suggesting the presence of wild type virus however, all plaques appeared to display green fluorescence i.e. 'white' wild type plaques were not observed. Speculation included the possibility that the green virus only produced plaques when some form of attenuated helper virus (slow growing or unable to form plaques) was present. To test this theory limiting dilutions of green virus at 0.4 pfu/well were plated out on BHK monolayers in 96 well plates. All plaques observed exhibited green fluorescence. DNA was extracted from plaque positive and negative wells four days post infection and PCR was done to detect wild type and recombinant gp150. If the recombinant virus was able to enter cells (via gp150 supplied in *trans* by growth with a helper virus), replicate, but was not able to spread without helper virus it may be possible to detect independent recombinant virus by PCR. That is to say, wells in

Figure 3.1.5. PCR analysis to check for the presence of wild type MHV-68 gp150 DNA in green fluorescent 'gp150 knockout' MHV-68 clones



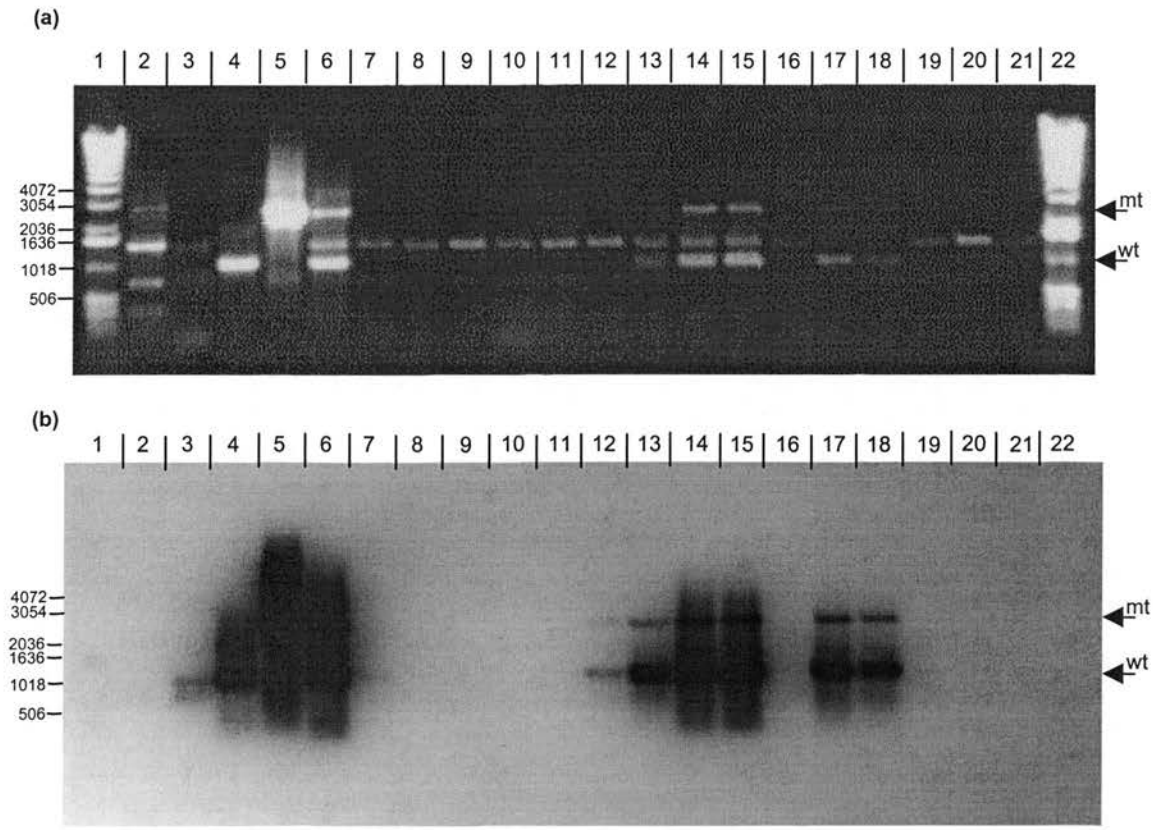
To generate a recombinant gp150 knock out (KO) virus MHV-68 genomic DNA and pgp150/gfp-C1 were cotransfected into BHK cells. Homologous recombination resulted in the production of viruses expressing green fluorescent protein (GFP). Plaque purification was done until all plaques observed exhibited green fluorescence. In order to determine if the consistently green fluorescent plaques were free of wild type virus, DNA was extracted five days post infection of BHK cells. Polymerase chain reaction (PCR) analysis, 25 cycles, was done using 0.5µg and 0.05 µg of extracted DNA with primers PAG11 and MA2 which flank the site in *gp150* of GFP gene cassette insertion. The predicted size of product amplified from the designed recombinant construct was 2.68kbp, and 1059bp from wild type *gp150*. The arrows indicate the position of these products - mt and wt respectively. The numbers on the left indicate the size of the1kbp DNA ladder marker bands in bp.

which no plaques were observed may have recombinant virus present alone which have undergone one round of replication. This assay aimed to test the theory that plaques occur only where helper virus with wild type gp150 and recombinant virus co-localise. DNA samples extracted from 16 randomly selected wells of a 96 well plate were analysed by 25 cycles of PCR, using primer pair PAG11 and MA2 (for primers and PCR conditions see section 2.1.8). By PCR, the wild type and recombinant product always co-localised and correlated precisely to the wells which were observed to have 'green' virus present i.e. plaques expressing GFP were seen (see figure 3.1.6.a). Only those wells which displayed green fluorescing cells were positive by PCR. To improve sensitivity and to detect virus specific bands (the PCR was prone to non specific products – also present using BHK DNA alone as seen in figure 3.1.6.a) the PCR products, following agarose gel electrophoresis, were transferred to a nylon membrane. The membrane was probed with a ^{32}P radiolabelled probe generated by random primed labelling using the entire gp150 gene as a template. As figure 3.1.6.b shows consistent co-localisation of recombinant and wild type PCR products was detected, neither product was observed independent of the other. Lanes of the Southern blot that appeared negative after 24 and 48 hour autoradiographic film exposure were cut from the blot and exposed to autoradiographic film independent of the positive lanes for longer periods of time (up to two weeks), no further bands were detected.

3.1.4 Southern Blot Analysis

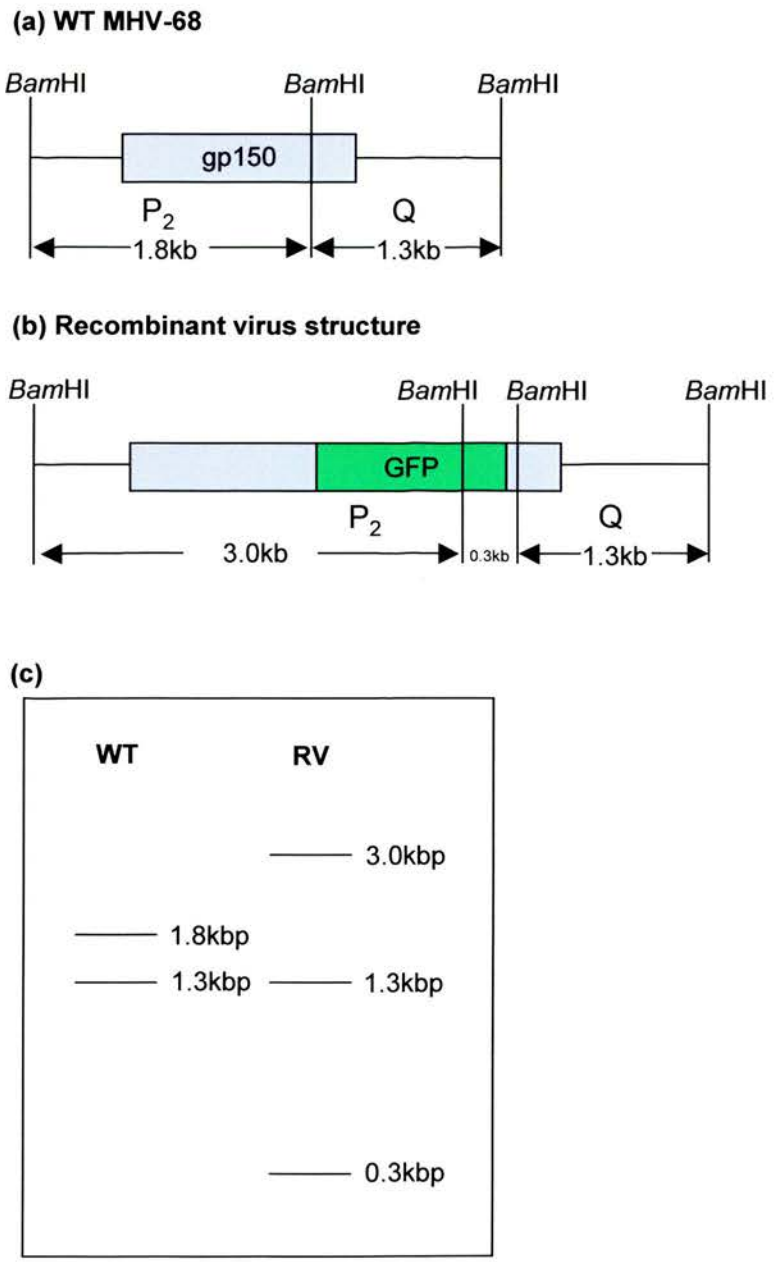
In order to determine if homologous recombination had occurred at the required site restriction digestion of DNA extracted from 'green' virus cultures was carried out using *Bam*HI. *Bam*HI was selected for use as there is a *Bam*HI site within gp150 and the *Bam*HI sites flanking this site produce gp150 positive bands of a relatively small and identifiable size, 1.3kbp and 1.8 kbp, upon digestion The digests were separated by agarose gel electrophoresis and transferred to a nylon membrane. The Southern blot was probed with a ^{32}P radiolabelled probe generated by random primed labelling using the whole gp150 gene, cut from the plasmid pSP72/gp150 using *Hin*dIII and *Eco*RI and electroeluted from an agarose gel, as a target. Figure 3.1.7. shows the *Bam*HI restriction maps of the gp150 encoding region within the MHV-68

Figure 3.1.6. PCR analysis to check for the presence of wild type MHV-68 gp150 DNA in green fluorescent 'gp150 knockout' MHV-68 clones



In order to determine if wild type and recombinant virus gp150 DNA were consistently co-located in wells containing plaques expressing green fluorescent protein and if any gp150 DNA was present in wells negative for plaques in a 96 well plate of limiting dilutions (0.4pfu/well) of 'green fluorescent' recombinant virus a polymerase chain reaction (PCR) analysis was done. DNA was extracted from 16 random wells, PCR, 25 cycles, was done using primers PAG11 and MA2 which flank the site in *gp150* of GFP gene cassette insertion. The predicted size of product amplified from the designed recombinant construct was 2.68kbp (mt), and 1059bp from wild type (wt) *gp150*. (a) Products were separated by electrophoresis through a 0.7% agarose gel containing ethidium bromide and visualised using a UV transilluminator. (b) In addition, products were transferred from the agarose gel to a nylon membrane by capillary transfer. The nylon membrane was probed with a ^{32}P labelled gp150 DNA probe in order to distinguish gp150 specific products. The probe was generated by random primed labelling using the entire gp150 gene as a target. The membrane was exposed to autoradiographic film for 24 hours. Lanes: 1 & 22 - 1 kb DNA ladder, 2 & 3 - BHK DNA, negative control, 4 - wild type MHV-68 DNA, 5 - pgp150-gfp-C1, 6 to 21 - wells 1 - 16. The arrows indicate the position of the gp150 products mt and wt. The numbers on the left indicate the size of the1kbp DNA ladder marker bands in bp.

Figure 3.1.7. Predicted *Bam*HI restriction maps of the gp150 encoding region of the MHV-68 genome in wild type and recombinant virus



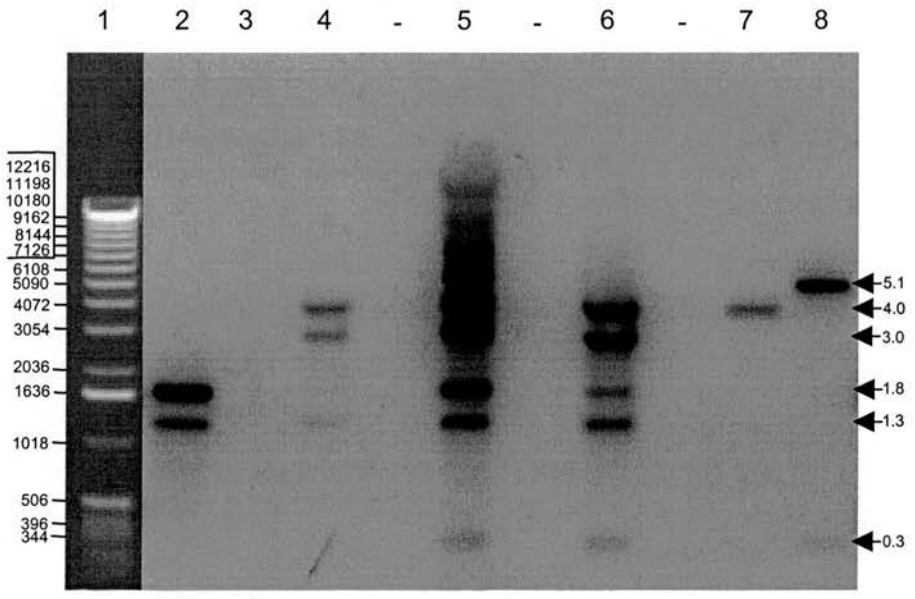
Predicted *Bam*HI restriction site maps of the gp150 encoding region of the MHV-68 genome for (a) wild type and (b) gp150 'knock out' recombinant virus. Letter designation of *Bam*HI fragments in the wild type MHV-68 genome are in accordance with Efstathiou *et al.*, 1990. (c) Schematic representation of the predicted bands detected when probing *Bam*HI restriction digests of wild type MHV-68 (WT) and gp150 'knock out' recombinant virus (RV), separated by agarose gel electrophoresis and transferred to a nylon membrane, using a gp150 specific random primed ^{32}P radiolabelled probe.

wild type and predicted recombinant genome indicating the sizes of bands predicted to be observed following the required double crossover homologous recombination event.

The Southern analysis of *Bam*HI digests in figure 3.1.8. shows the presence of wild type *Bam*HI restriction fragments (1.3kbp and 1.8kbp) in the 'green' virus samples. In addition, there are bands of ~0.3kbp, 3kbp and 4kbp, from the banding pattern it appears that the whole plasmid may have integrated into the genome adjacent to the 5' end of gp150 most likely via a single recombination event. The inserted plasmid could most probably loop out of the recombinant MHV-68 genome returning the genome to wild type. The relative intensity of the 1.3kbp band for recombinant virus lanes is high in comparison to larger bands. This may be explained by the theory that both the wild type genome and recombinant genome with the whole plasmid integrated are predicted to produce a 1.3kbp fragment positive for gp150 DNA following digestion with *Bam*HI. Figure 3.1.9. illustrates the integration event which could explain this out come.

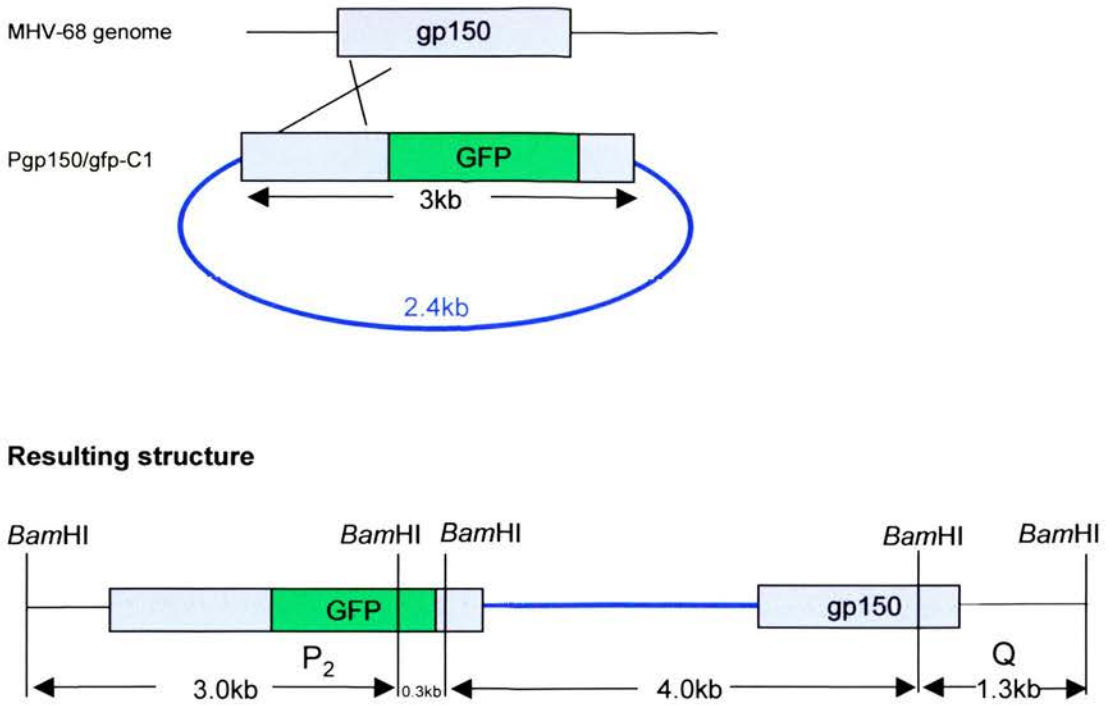
In summary recombinant viruses, expressing GFP were generated however the targeted gp150 gene had not been disrupted. The entire plasmid appears to have integrated into the genome which would explain the co-localisation of wild type and recombinant gp150 detected by PCR. This is unlikely to be a useful reagent as a GFP tagged 'wild type' virus because the plasmid insertion is likely to be unstable. The plasmid insert may loop out and the virus resort back to complete wild type virus – the detection of wild type *Bam*HI digestion fragments suggests this may be occurring. After five passages of the 'green' viruses no wild type plaques were observed however, on further passage wild type virus may re-emerge at a level detectable by plaque assay.

Figure 3.1.8. Restriction digestion, using *Bam*HI, of DNA from three different clones of 'green fluorescent' recombinant virus



DNA was extracted from BHK cells infected with 'green fluorescent' recombinant virus (RV) clones (generated by homologous recombination of MHV-68 DNA and vector pgp150/gfp-C1) and digested with *Bam*HI. All plaques induced by these viruses expressed green fluorescent protein i.e. no wild type plaques were observed. Restriction digests of DNA were electrophoresed through a 0.7% agarose gel and transferred to a nylon membrane by capillary transfer. The membrane was probed using a gp150 specific ^{32}P radiolabelled probe. The probe was generated by random prime labelling using the whole gp150 gene as a target. The membrane was exposed to autoradiographic film for 7 days. Lane: 1- 1kb DNA ladder, 2 - wild type MHV-68 *Bam*HI digested DNA, 3 - BHK DNA, 4 - gp150 RV 4E, 5 - gp150 RV 6B, 6 - gp150 RV 6F, 7 - pSP72/gp150 *Bam*HI digested (4kbp), 8 - pgp150/gfp-C1 *Bam*HI digested, dashes indicate blank lanes. The numbers on the left indicate the size in bp of the 1kb DNA ladder marker bands. Numbers on the right indicate the approximate size of predominant bands in kbp.

Figure 3.1.9. Schematic representation of a single recombination event resulting in integration of the vector pgp150/gfp-C1 into the MHV-68 genome



The diagram shows the homologous recombination event and resulting arrangement of the gp150 region of the MHV-68 genome which is likely to have occurred - determined from the size of *Bam*HI restriction digest fragments, positive for gp150 sequence, for 'green' recombinant virus DNA (bands of 4.0, 3.0, 1.8, 1.3 and 0.3kbp). It appears that the whole plasmid may have inserted into the MHV-68 genome via a single homologous recombination event. The detection of a 1.8kb fragment is likely to be produced from digestion of wild type MHV-68 which occurs when the plasmid loops out of the genome. This may explain why the 1.3kbp is more intense relative to larger bands for 'green' recombinant viruses in figure 3.1.8. as both wild type and recombinant genomes produce a 1.3kbp (Q) fragment upon *Bam*HI digestion.

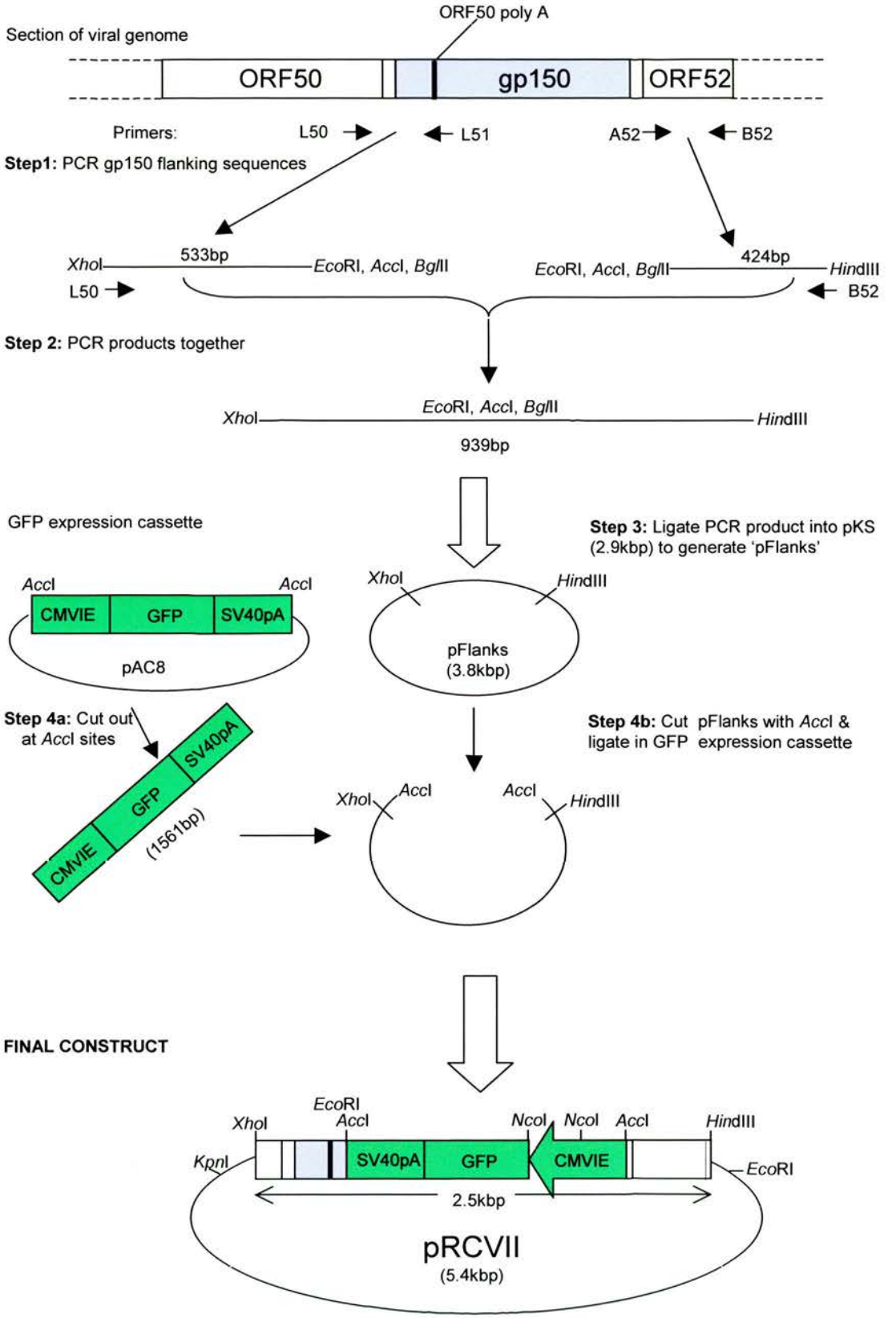
3.1.5 Recombinant Virus II

In order to produce a gp150 'knock out' recombinant virus a new plasmid construct was generated. This new construct was designed to be compatible with a gp150 complementary cell line i.e. the possibility of the knock out virus reverting to wild type by recombining with the gp150 DNA pertained by the cell line was minimised. As yet it was not known if gp150 was essential to virus replication however, the absence of gp150 could have adverse effects on virus growth therefore it was considered worthwhile to take this precautionary measure. This was done by limiting, as far as possible, sequences in the recombinant vector that were complementary to the gp150 gene which would be carried by a complementary cell line.

Figure 3.2.1. illustrates the strategy, described here, used to generate the recombinant virus construct, named pRCVII. Initially the sequences flanking gp150 were amplified by PCR. The left hand flanking sequence, of approximately 500bp, was obtained using primers L50 and L51 (for primers and PCR conditions refer to section 2.1.8). Primer L51 is positioned within the N terminal region of *gp150*, ideally the sequences used for targeting homologous recombination would be out with the gp150 gene so that propagation of the recombinant virus in a gp150 complementary cell line would not allow wild type revertants to form via recombination of the recombinant virus genome with the gp150 gene encoded by the cell line. However, at the 5' end of the gp150 gene there is a polyadenylation consensus sequence for ORF 50 (Mackett *et al.*, 1997) (refer to figures 3.2.1. and 1.8 for location), elimination of this may alter the viral phenotype in addition to any effects caused by the absence of gp150 and so this region was included in the recombinant construct. Primers A52 and B52 were used in a PCR to generate the right hand flanking sequence of approximately 400bp (for primers and PCR conditions refer to section 2.1.8). The 5' primer, A52 is located just downstream of the gp150 gene (refer to figure 3.2.1.).

Primers L51 (antisense) and A52 (sense) as shown in section 2.1.8 were designed with three complementary restriction enzyme sites at their 5' ends. In the conditions

Figure 3.2.1. Strategy used to generate pRCVII, the construct for generation of a gp150 negative recombinant MHV-68 virus



of a PCR, the products of L50/L51 and A52/B52 could anneal to each other via these complementary sequences and act as a target for generation of a product consisting of the two flanks joined (see figure 3.2.1). Therefore, the initial PCR products were purified and then 1µl of each product was used as the combined target in a subsequent PCR using primers L50 and B52 resulting in a product of 965bp (for primers and PCR conditions refer to section 2.1.8). The product was purified and then digested using *Xho*I and *Hin*dIII. The cut product was ligated into the cloning vector pSK (2.9kbp) which had been prepared by digestion with *Hin*dIII and *Xho*I followed by dephosporylation to prevent self ligation. XL1-Blue *E. coli* were transformed with the ligation mix using the heat shock method. Of 78 transformants which grew, selected using ampicillin, 12 were picked, grown up and their DNA extracted. Digestion of the DNA using *Eco*RI indicated that one colony contained pSK with the insert (two bands of approximately 3.4kbp and 400bp were generated, pSK alone only has one *Eco*RI site). A small scale DNA preparation, named pFlanks, of this clone was cut with *Acc*I and then dephosphorylated in preparation to insert a gene cassette encoding green fluorescent protein (GFP). The GFP gene cassette consisting of *gfp* flanked by the CMVIE promoter and the SV40pA was cut from vector pAC8 using *Acc*I. The cassette differed slightly from the same cassette in pEGFP-C1, used for the previous recombinant construct in that the multiple cloning site between *gfp* and the SV40 poly A signal in the pAC8 construct had been removed. The cassette was electroeluted from an electrophoresed agarose gel of the *Acc*I digestion of pAC8, purified and ligated into the *Acc*I cut pFlanks. The ligation mix was used to transform XL1-Blue *E. coli* by the heat shock method. Of 36 transformants, selected using ampicillin, 12 were picked, grown up and their DNA extracted. From restriction digestion of the DNA samples, using *Nco*I and *Eco*RI, 7 clones appeared to have the plasmid plus insert, producing bands of approximately 3.4kbp, 1kbp, 700bp and 251bp. This banding pattern indicated the GFP gene cassette was orientated in the opposite transcriptional direction to gp150. Preferentially the GFP cassette would be orientated in the same transcriptional direction as gp150 therefore the rest of the transformants (24 more) were picked and analysed as before to determine if any contained the GFP insert in this direction. The GFP cassette was orientated in the same direction as all the previous clones.

Rightwards transcriptional orientation of GFP was not essential therefore a large DNA preparation of one transformant was generated using the CsCl gradient method ready for production of the recombinant virus. This plasmid was named pRCVII.

In order to check that the GFP cassette was functional BHK cells were transfected with 30μg of either uncut pRCVII or *Kpn*I and *Hin*dIII digested pRCVII (this digestion cuts the GFP cassette plus flanking sequences from the plasmid, described as the recombinant cassette). Cells expressing green fluorescent protein were observed using UV microscopy 24 hours post transfection.

Subsequently, *Kpn*I/*Hin*dIII digested pRCVII was co-transfected with MHV-68 genomic DNA into C127 cells. C127 cells were used as opposed to BHK cells as they form more uniform monolayers, show greater contact inhibition of growth and the MHV-68 plaques are more discrete. The sequence for recombination with the viral genome, known as the 'recombinant cassette', was excised entirely from the vector by restriction enzyme digestion prior to transfection in order to prevent the whole plasmid integrating into the genome. Circularised plasmids are used for the production of recombinant viruses (Mackett *et al.*, 1984) however, linearised sequences were considered to be less prone to single recombination events resulting in insertion of the vector and failure to displace the targeted gene. Fully formed 'green fluorescent' plaques expressing GFP were observed 7 days post transfection, as were wild type 'white' plaques. Plaque morphology of 'green fluorescent' plaques did not differ in their appearance, i.e. in size and development rate, to wild type plaques. 'Green fluorescent' plaques were picked through the agar overlay of these cultures. Plaque purification of 'green fluorescent' virus was performed by passage of sequential plaque picks. Despite selection of 'green' plaques physically isolated from other plaques in monolayers wild type plaques were consistently observed at level of 1:15 to 1:5 of total plaques. Limiting dilutions of plaques (0.4pfu/well) were plated on cell monolayers in 96 well plates in order to isolate 'green' plaques. Upon subsequent plating of green plaques harvested from wells of limiting dilutions which appeared to have green plaques present in the absence of

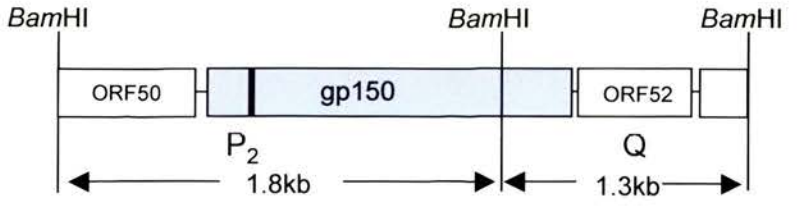
wild type plaques –green virus was observed however wild type plaques also developed albeit at a lower level i.e. 1:100.

Although a low level presence of wild type plaques (approximately 1:100) was present after six passages DNA was extracted for analysis to determine if the 'green' virus present had been generated by the required recombination event. The DNA from four green virus samples, each derived from the initial transfection, which had undergone plaque picking and limiting dilution, was extracted from T175 flasks of virus grown on C127 cells. *Bam*HI digests of the DNA were subjected to agarose gel electrophoresis followed by Southern analysis. The Southern blot was probed with a ^{32}P radiolabelled probe generated by random prime labelling using the whole gp150 gene as a target. In addition, a ^{32}P radiolabelled probe generated by random prime labelling using the GFP gene cassette DNA as a target was used. Figure 3.2.2 shows the predicted products resulting from a *Bam*HI digest of the designed recombinant virus.

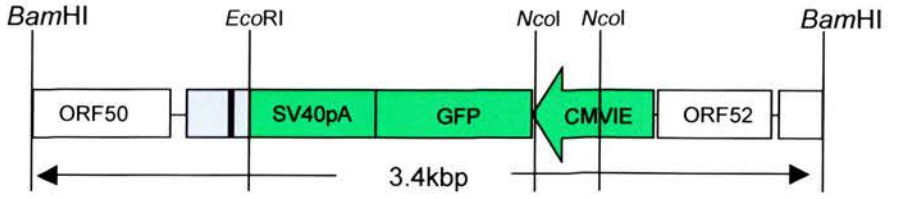
As figure 3.2.3 shows *Bam*HI digested wild type MHV-68 DNA probed with the gp150 specific probe produced the expected bands of 1.3kbp and 1.8kbp, with longer exposure times (>24 hours) of the probed blot to autoradiographic film two larger bands were observed of approximately 4kbp and 5kbp – these sizes correlate with *Bam*HI partial digestion products. These bands were seen for each of the four 'green' virus DNA samples, in addition there was a high molecular weight smear of approximately 14-18kbp for the 'green' virus DNA alone. When probing the same digests with the GFP specific probe the large smear alone was detected for the 'green' virus DNA (refer to figure 3.2.4). This suggested the recombinant cassette may have inserted into a region within one of the large *Bam*HI fragments of the MHV-68 genome. Two regions of the genome are known to produce large DNA restriction fragments when digested with *Bam*HI, these are described as fragment A_1 of approximately 18kbp and A_2 of approximately 14kbp (Efstathiou *et al.*, 1990b). As shown in figure 3.2.5a A_1 is located at the left hand end of the genome and A_2 is located to the right of the centre of the genome beginning at ~77kbp from the left of the genome. Insertion of the recombinant cassette into A_1 did not seem unreasonable

Figure 3.2.2. Predicted *Bam*HI restriction maps of the gp150 encoding region of the MHV-68 genome in wild type and recombinant virus generated using pRCVII

(a) WT MHV-68

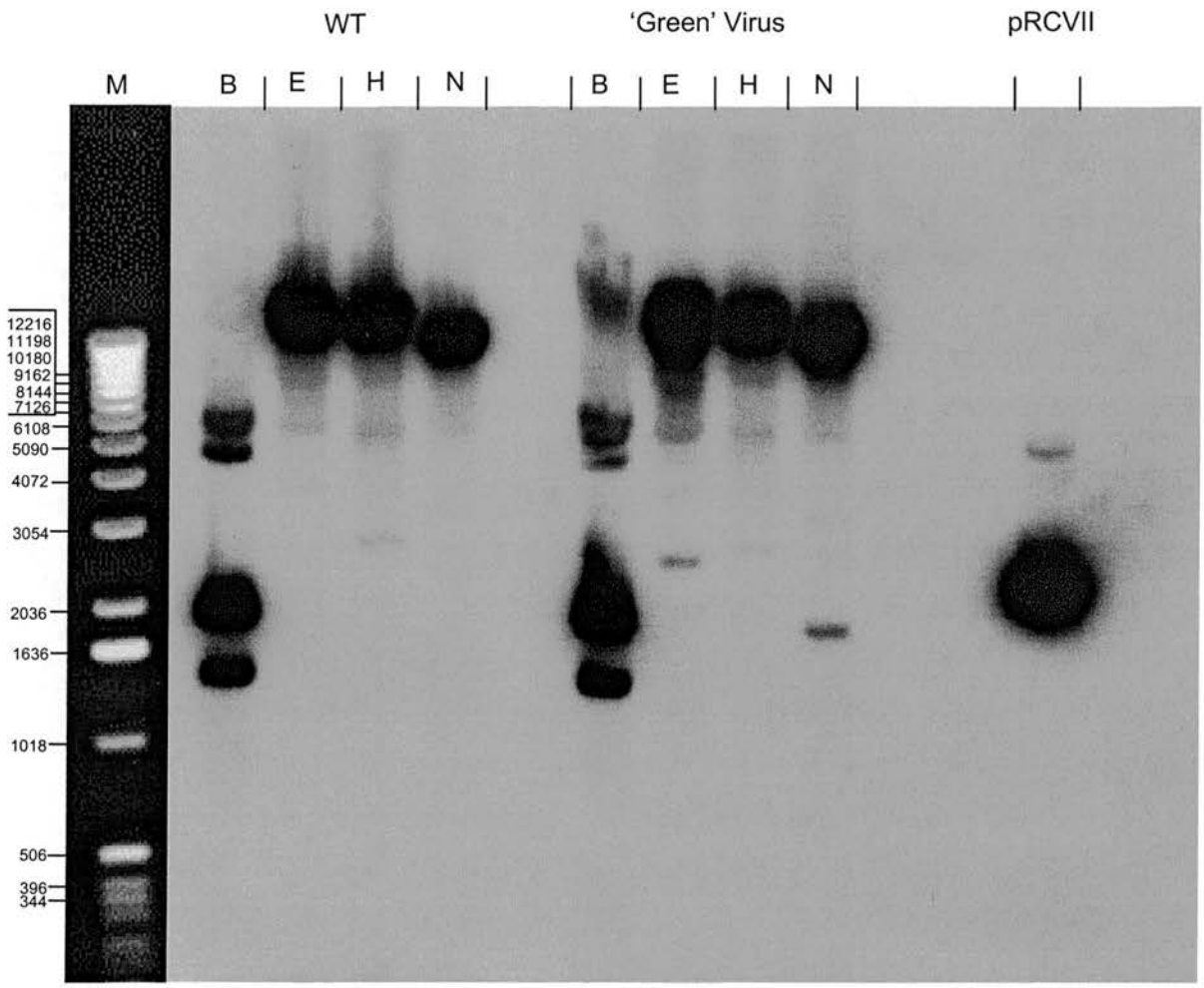


(b) Recombinant virus structure - generated using pRCVII



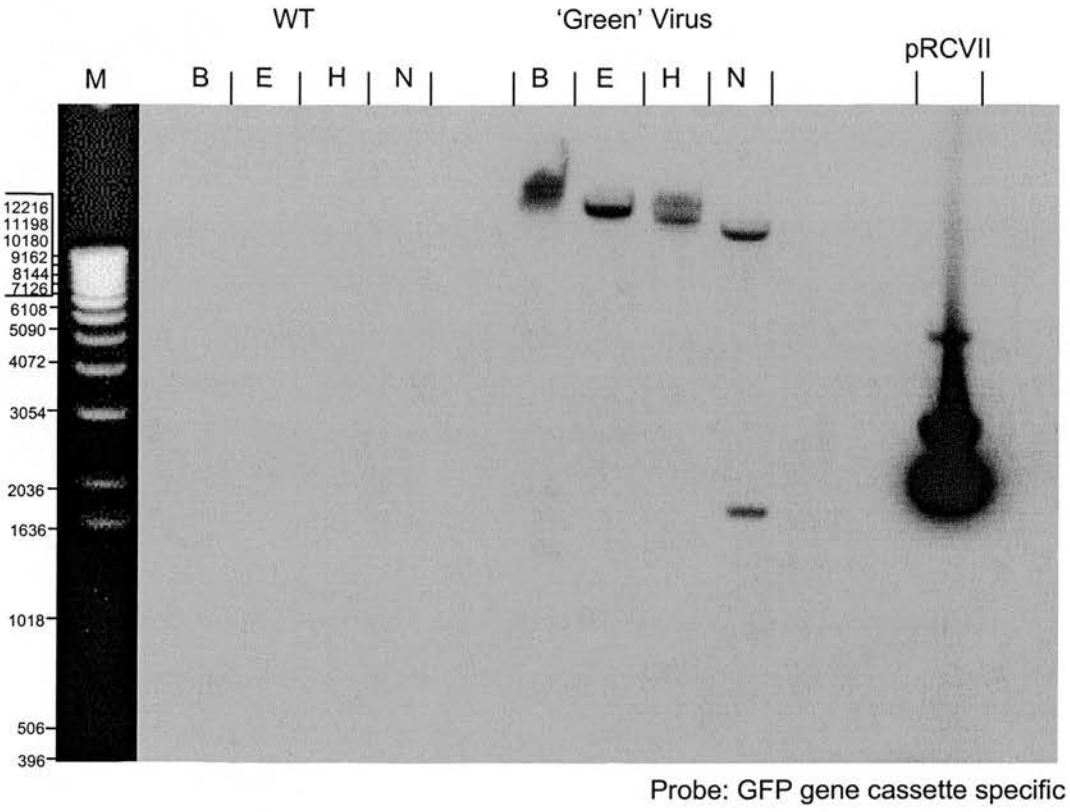
Predicted *Bam*HI restriction maps of the gp150 encoding region of the MHV-68 genome for (a) wild type (WT) virus and (b) a gp150 recombinant virus generated using pRCVII. Letter designation of the *Bam*HI fragments in the wild type MHV-68 genome are in accordance with Efstathiou *et al.*, 1990b.

Figure 3.2.3. Southern analysis of restriction digests of DNA from wild type MHV-68 and 'green fluorescent' recombinant virus generated using pRCVII



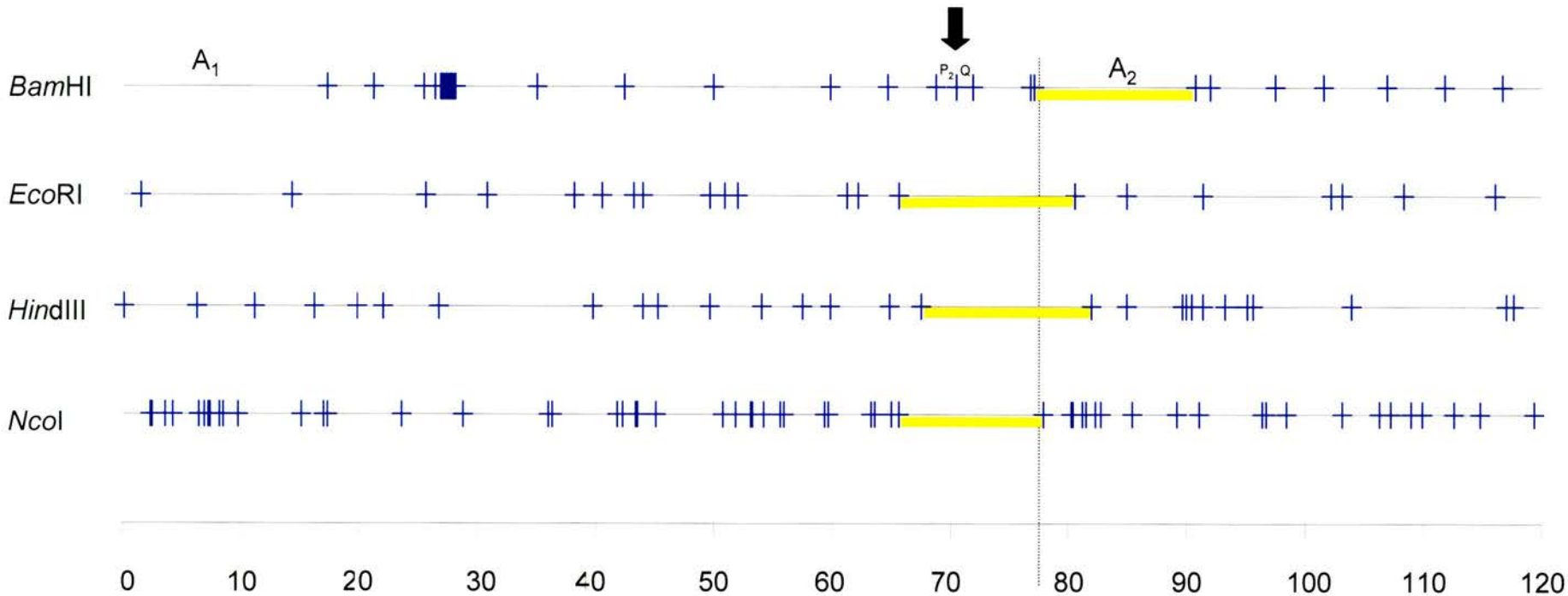
Representative Southern analysis. DNA was extracted from C127 cells infected with either wild type (WT) MHV-68 or 'green' virus generated using pRCVII. DNA samples were digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) or *Nco*I (N) and electrophoresed through a 0.7% agarose gel and transferred to a nylon membrane. The Southern blot was probed with a ^{32}P radiolabelled gp150 specific probe generated by random primed labelling using the whole gp150 gene as a target. The probed blot was exposed to autoradiographic film for 24 hours. The numbers on the left indicate the size of 1kb DNA ladder marker (M) bands in bp. The far left lane contains the vector pRCVII (5.4kbp) partially digested with *Kpn*I and *Xho*I (this cuts the recombinant cassette (~ 2.5kbp) from the plasmid (2.9kbp)). Note: the large patches at >12kbp for WT and 'Green' virus lanes E, H and N appeared as single bands, similar for those seen in figure 3.2.5., with a shorter autoradiographic film exposure time of 6 hours however a longer exposure time is shown here in order to achieve better resolution of the smaller bands in the 'Green' virus lanes E and N.

Figure 3.2.4. Southern analysis of restriction digests of DNA from wild type MHV-68 and 'green fluorescent' recombinant virus generated using pRCVII



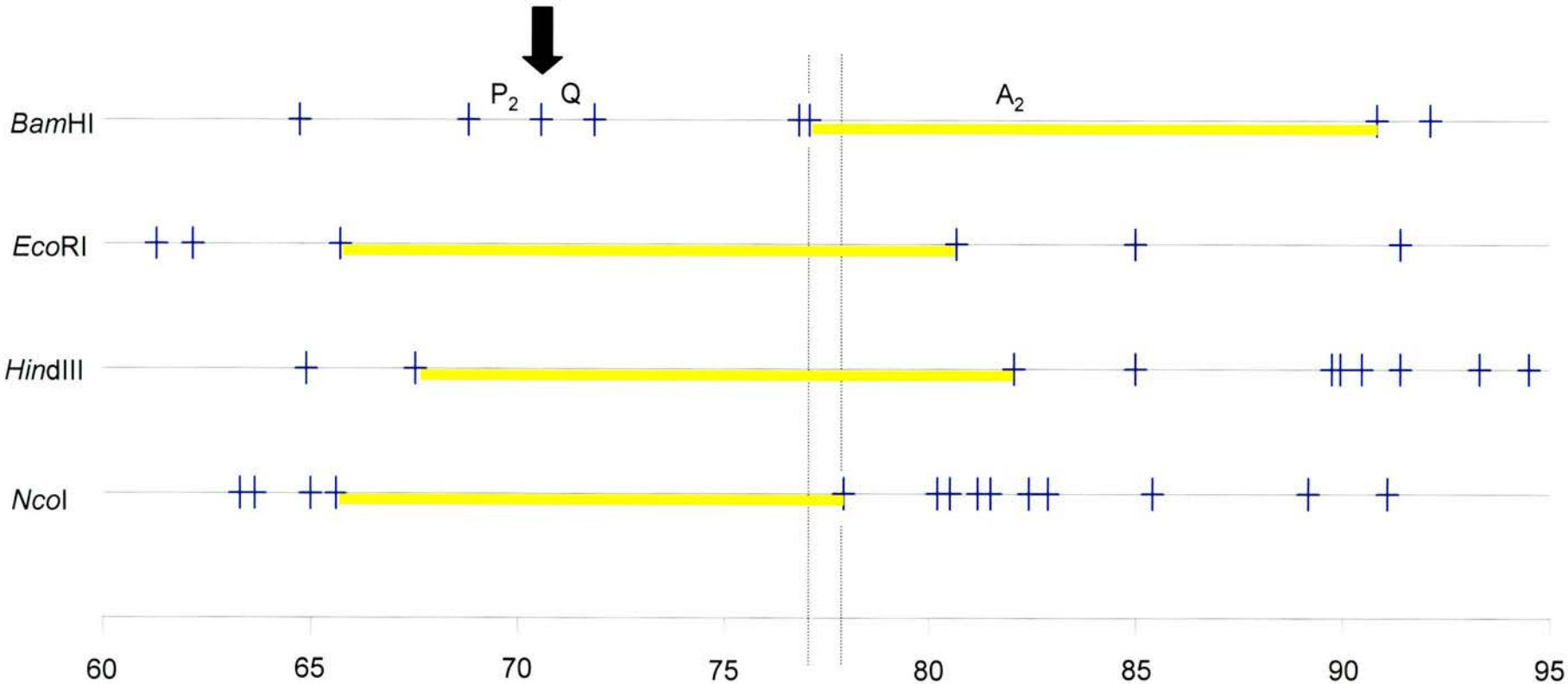
DNA was extracted from C127 cells infected with either wild type (WT) MHV-68 or 'green' virus generated using pRCVII. DNA samples were digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H) or *Nco*I (N) and electrophoresed through a 0.7% agarose gel and transferred to a nylon membrane. The Southern blot was probed with a ^{32}P radiolabelled green fluorescent protein (GFP) gene cassette specific probe generated by random primed labeling using the whole GFP gene cassette as a target. The probed blot was exposed to autoradiographic film for 24 hours. The numbers on the left indicate the size of the 1kb DNA ladder marker (M) bands in bp. The far left lane contains the vector pRCVII (5.4kbp) digested with *Kpn*I and *Xho*I (this cuts the recombinant cassette (~ 2.5kbp) from the plasmid (2.9kbp)).

Figure 3.2.5a. Possible insertion site of the recombinant cassette determined from Southern analysis of restriction digests of viral DNA using *Bam*HI, *Eco*RI, *Hin*dIII and *Nco*I



*Bam*HI, *Eco*RI, *Hin*dIII and *Nco*I restriction maps of the MHV-68 genome. Based on the restriction fragments, from digests of 'green' viral DNA using different restriction enzymes, that were detected in Southern analysis using probes for gp150 and the green fluorescent protein encoding cassette, the recombinant virus cassette is thought to have inserted into the viral genome in the region marked by the dotted line. Numbers indicate the region of the MHV-68 unique DNA in kb according to Virgin *et al.*, 1997. Restriction sites along the genome are indicated by the blue vertical lines, these are in scale with one another. See figure 3.2.5b for close up of the putative region of insertion. The arrow indicates the targeted site for recombination i.e. the location of the genomic gp150 gene across *Bam*HI fragments P_2 and Q. A_1 and A_2 are the large *Bam*HI restriction fragments as designated by Efstathiou *et al.*, 1990b. The yellow highlighted areas indicate large restriction fragments >12kbp.

Figure 3.2.5b. Possible insertion site of the recombinant cassette determined from Southern analysis of restriction digests of viral DNA using *Bam*HI, *Eco*RI, *Hin*dIII, and *Nco*I



*Bam*HI, *Eco*RI, *Hin*dIII and *Nco*I restriction maps of the MHV-68 genome. Based on the restriction fragments, from digests of 'green' viral DNA using different restriction enzymes, that were detected in Southern analysis using probes for gp150 and the green fluorescent protein encoding cassette, the recombinant virus cassette is thought to have inserted into the viral genome within the region marked by the dotted lines. Numbers indicate the region of the MHV-68 unique DNA in kb according to Virgin *et al.*, 1997. Restriction sites along the genome are indicated by the blue vertical lines, these are in scale with one another. The arrow indicates the targeted site for recombination i.e. the location of the genomic gp150 gene across *Bam*HI fragments P_2 and Q. A_2 is one of the large *Bam*HI restriction fragments as designated by Efstathiou *et al.*, 1990b. The yellow highlighted areas indicate large restriction fragments >12kbp.

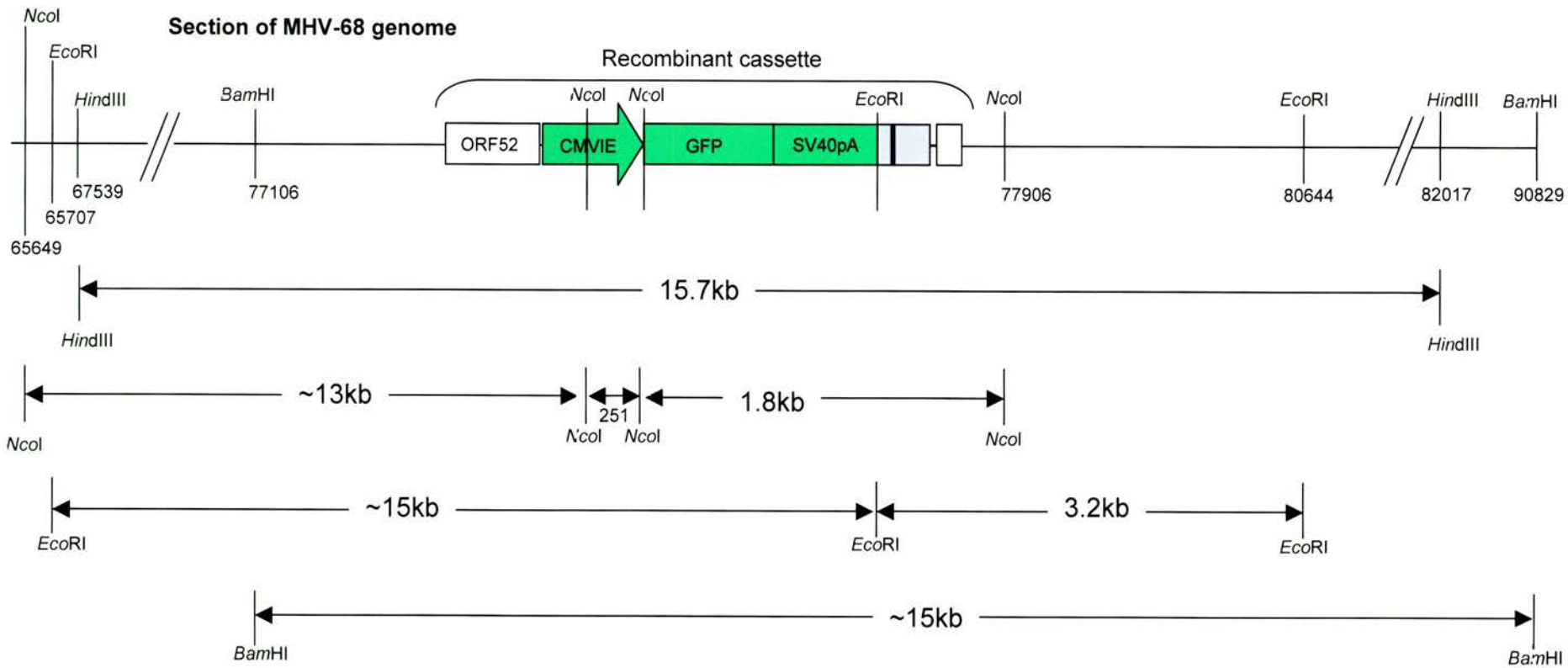
due to the experience of laboratory co-workers who were making MHV-68 recombinant viruses in the same manner, targeting other genes i.e. the bcl-2 and IL-8^R homologues (for genomic positions see figure 1.3). Their recombinant constructs commonly integrated into the left hand end of the viral genome rather than the targeted site.

To investigate if insertion into the left hand end of the genome was the case further digests of the 'green' virus DNA were done using *Hin*dIII, *Eco*RI and *Nco*I. *Hin*dIII and *Nco*I were selected as these have several sites at the left hand end of the genome across the region of the A_1 *Bam*HI fragment, if the insert was positioned in this region smaller bands, of a size easier to determine, should be detected. An *Eco*RI digest was also done as the recombinant cassette has an *Eco*RI site and so introduction into the genome would result in an extra *Eco*RI fragment being generated. *Nco*I was also chosen for this reason, within the recombinant cassette there are two *Nco*I sites.

Figure 3.2.3 shows the bands observed when digests were probed with the gp150 specific probe. For *Hin*dIII the appearance of the digest of 'green' virus DNA did not appear significantly different to that of wild type DNA. A large band was detected of ~14kbp, this indicated that the insert was not likely to be in the left hand end of the genome as the sizes of *Hin*dIII fragments range between 5 – 7kbp across the A_1 *Bam*HI fragment region (see figure 3.2.5a). The fainter smaller bands in the *Hin*dIII digests shown in figure 3.2.3 were only observed at long exposure times (≥24 hours) of the blot to autoradiograhic film and are likely to be background. For the *Eco*RI 'green' virus digest two bands were observed one very large band of ~13kbp and another of ~2.8kbp suggesting insertion of the recombinant cassette into an end of one of the large *Eco*RI restriction fragments of MHV-68. For wild type virus, just one large gp150 positive band was seen upon *Eco*RI digestion. Furthermore, two bands were detected in a *Nco*I digestion of 'green' virus DNA – one very large band of ~ 14kbp and another of ~ 1.5kbp. As figure 3.2.4 shows this was also observed when the same digest was probed using the GFP specific probe indicating that the recombinant cassette had integrated into a large *Nco*I fragment of

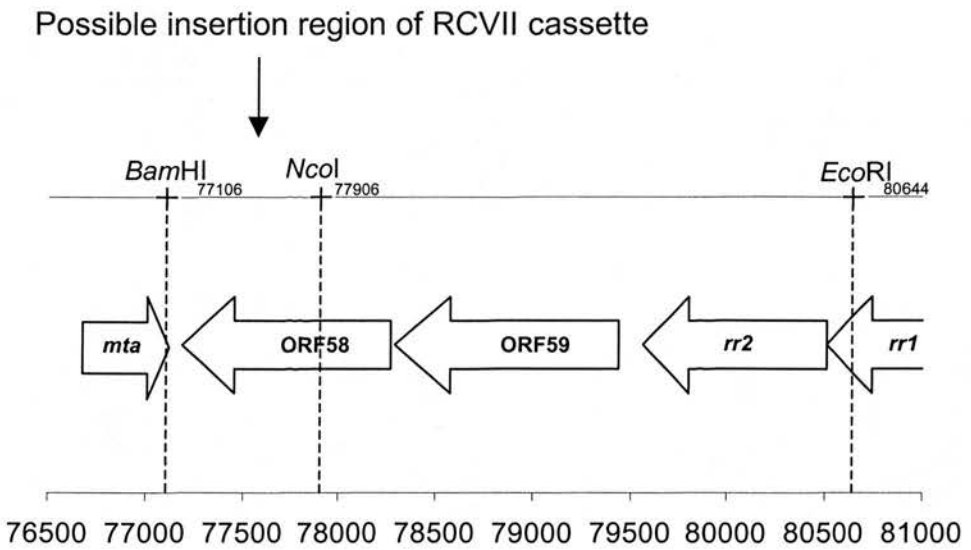
the viral genome, close to one end of the fragment. This resulted in the generation of a still large *Nco*I fragment and a smaller fragment produced from the cleavage of the new *Nco*I site and the *Nco*I site at the insertion end of the fragment. Restriction digest maps of the genome were compared to locate where large fragments produced by each restriction enzyme overlapped. To the right of the centre of the genome an area of 801bp was identified across which large digest fragments (>12kbp) for each of the four restriction enzymes used overlap. Refer to figures 3.2.5a. and 3.2.5b. this is the area in which the insert may be located. Figure 3.2.6 provides a schematic representation to explain how the bands detected by Southern analysis correlate to this proposed location of recombinant cassette integration. The smaller *Eco*RI fragment detected using the gp150 specific probe on the 'green' virus digest is slightly smaller (~2.8kb compared to 3.2kb) than the predicted band this could be due to a deletion of a portion of sequence. Figure 3.2.7 shows the genes located in this region i.e. ORF57 and ORF58. The data suggest the recombinant cassette has inserted into ORF58 and it is possible that a portion of ORF58 or a region upstream up to the next *Eco*RI restriction site may have been deleted. The function of ORF58 is not known, ORF57 encodes an immediate early transcriptional activator.

Figure 3.2.6. Possible explanation of bands observed in Southern analysis of different restriction digests of 'green' virus DNA



Schematic representation of possible origin of restriction fragments generated and detected in Southern analysis of *Bam*HI, *Eco*RI, *Hin*dIII and *Nco*I restriction digests of 'green' virus DNA. The numbers by the restriction sites indicate their position along the unique DNA of the MHV-68 genome according to the MHV-68 sequence submitted to Genbank by Virgin *et al.*, 1997.

Figure 3.2.7. Arrangement of ORFs in the region of the MHV-68 genome where the RCVII cassette may have integrated



Based on the restriction fragments, from digests of 'green' viral DNA using different restriction enzymes, that were detected in Southern analysis using probes for gp150 and the green fluorescent protein encoding cassette, the recombinant virus cassette is thought to have inserted into the viral genome within the region between the *Bam*HI and *Nco*I restriction sites indicated. Numbers indicate the region of the MHV-68 unique DNA in base pairs according to Virgin *et al.*, 1997. Open reading frames (ORFs): *mta* - M immediate early transcriptional activator, ORF58 - unknown function, ORF59 - DNA replication proten, *rr2* - small ribonucleotide reductase, *rr1* - large ribonucleotide reductase.

3.1.6 Earlier Analysis of Recombination by PCR

In view of the time taken to purify and analyse recombinant viruses a system using PCR was designed to determine if the desired recombination event had occurred soon after the initial transfection stage. Following co-transfection of MHV-68 DNA and *Kpn*I and *Xho*I cut pRCVII BHK cells were plated into wells of a 24 well plate following co-transfection with. DNA was extracted from each well five days post transfection and PCR analysis was done to determine if the required recombination event had occurred. For this, diagnostic primers were used i.e. one primer within the GFP cassette, SV40d and another out with the recombinant cassette RCVd, positioned upstream of the recombination site (for primers and PCR conditions refer to section 2.1.8). Using this primer pair a product of 626bp should be observed if the specific recombination event has occurred. Control PCRs for the detection of viral DNA in the samples using primers L50 and L51 for the left hand region of gp150 were positive, producing the predicted product of approximately 533bp. In contrast, a product was not detected by a 40 cycle PCR using the diagnostic primers. This suggested the desired homologous recombination had not occurred using these conditions.

3.1.7 Mammalian Expression of Gp150

Attention was directed to generating a mammalian cell line able to express gp150. In the event that gp150 is essential for MHV-68 replication a cell line able to express gp150 would be an invaluable tool as a complementary cell line in which to grow a gp150 negative recombinant virus. Initial data suggested the first recombinant virus engineered may require a helper virus for replication and so a cell line able to supply gp150 in *trans* would allow propagation and purification of the recombinant virus.

3.1.8 pBabePuro for Mammalian Expression of Gp150

The design of the gp150 complementary cell line aimed to avoid potential problems of toxicity of glycoprotein expression and so *gp150* was placed under the control of the putative MHV-68 gB promoter PgB. Toxicity is a property of glycoproteins known to hinder the generation of constitutively expressing cell lines (Whang *et al.*,

1987, Conway *et al.*, 1989, Motz *et al.*, 1987, Gompels & Minson, 1989). For example Whang *et al.*, 1987, experienced rapid loss of cells expressing gp340/220 consistent with the theory that high expression of glycoproteins is detrimental to cell growth. In theory, cells containing *gp150* under the control of PgB would express gp150 upon infection with MHV-68, which would provide the necessary viral transactivation signals to act on PgB and induce gp150 expression. In the absence of virus infection gp150 expression should be minimal and present in substantial amounts only when required following virus infection. A similar system was used to successfully propagate a gH negative HSV-1 mutant in Vero cells carrying the HSV-1 gH gene under the control of the HSV-1 gD promoter (Forrester *et al.*, 1992).

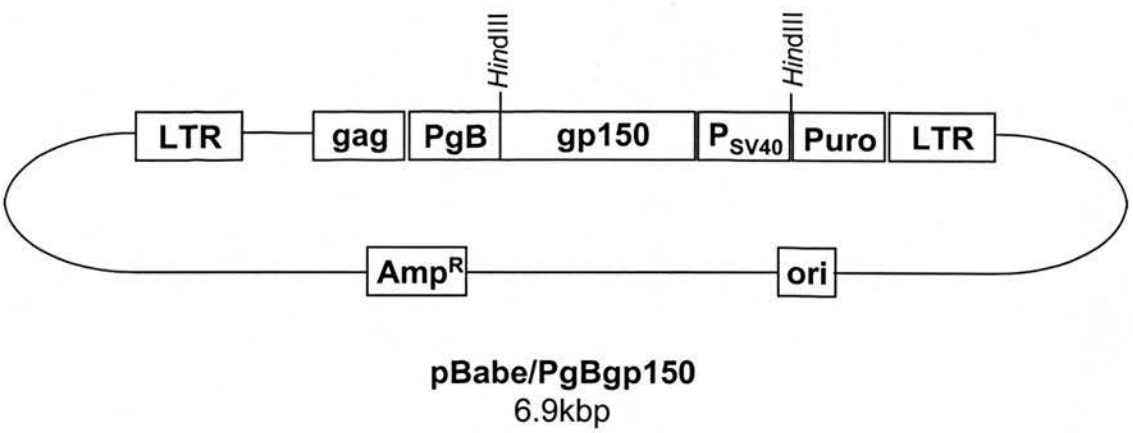
Primers PGB1 and PGB2 were used in a PCR to amplify a 265bp region directly upstream from the translation initiation site of gB on the MHV-68 genome (Stewart *et al.*, 1994) (for primers and conditions see section 2.1.8). This is the putative gB promoter (PgB). The PCR product was cut with *Xho*I and *Hin*dIII and ligated into pSP72 (previously cut with *Xho*I and *Hin*dIII and dephosphorylated ready for ligation). The ligation mix was used to transform XL1-Blue *E. coli* by the heat shock method. Of 350 colonies selected using ampicillin 12 were picked, grown up and their plasmid DNA extracted. Extracted DNA was analysed by restriction digestion with *Xho*I and *Hin*dIII, 8 samples appeared to have the insert (two bands, one of ~2.4kbp and another of ~265bp correlating to the plasmid and insert respectively). The plasmid DNA from one sample, named pSP72/PgB was cut with *Hin*dIII and *Eco*RI and then dephosphorylated in preparation to receive a gp150 DNA insert. The entire gene encoding gp150 was amplified by PCR using primers IA3 and IA4 (see section 2.1.8 for primers and PCR conditions). The product, ~1.5kbp was cut with *Hin*dIII and *Eco*RI and then ligated into pSP72/PgB. A 1µl sample of the ligation mix was used as the target in a PCR with primers PGB1 and IA4 in order to amplify a product consisting of gp150 downstream of PgB i.e. PgBgp150 (for primers and conditions see section 2.1.8). The identity of the 1.8kbp product was checked by digestion with *Hin*dIII which correctly produced two bands of ~1.5kbp and ~265bp correlating to gp150 and PgB, respectively. PgBgp150 was cut with

*Bgl*II and *Eco*RI and ligated into the Moloney murine leukemia virus based mammalian expression vector pBabePuro (Morgenstern & Land, 1990) (previously cut with *Bam*HI and *Eco*RI and dephosphorylated ready to receive the insert). The ligation mix was used to transform XL1-Blue *E. coli* using the heat shock method. All 21 colonies that grew, selected using ampicillin, were picked, grown up and their plasmid DNA extracted. Plasmid DNA was digested using *Hin*dIII to test for the presence of the insert, only one clone produced the correct banding pattern (two bands, one of 5kbp and another of 1.9kbp). A large scale plasmid preparation was made from this clone, the plasmid was named pBabe/PgBgp150. Figure 3.2.8 shows the structure of pBabe/PgBgp150. Complete integrity of the PgBgp150 region of the plasmid was confirmed by sequence analysis using the gp150 spanning sequencing primers and primer PGBseq (refer to section 2.1.8 for primers).

3.1.9 Generation of a Cell Line Carrying pBabe/PgBgp150

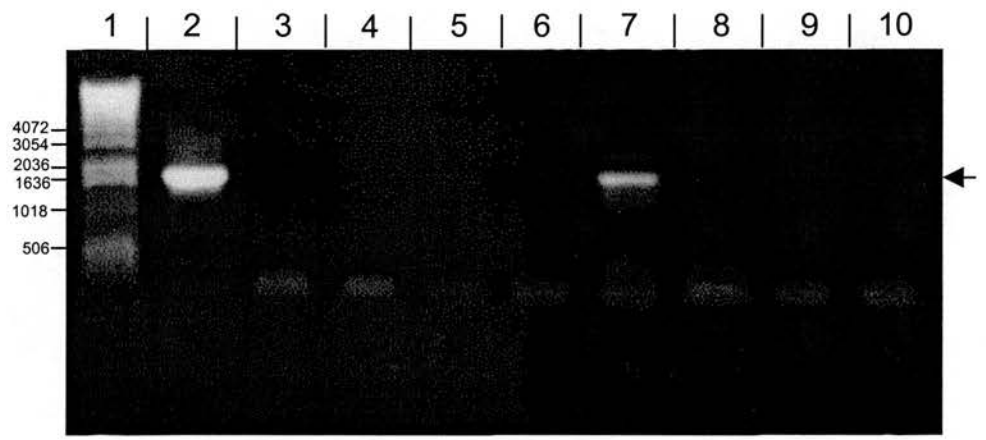
The vector pBabe/PgBgp150 was transfected into C127 cells by electroporation. In addition the vector pBabePuro was transfected into cells in order to generate a control cell line i.e. vector without PgBgp150 cassette. Following transfection, cells were plated into wells of a six well plate. The culture media was removed 24 hours post transfection and selective media containing puromycin was added. After three days under puromycin selection resistant colonies of cells emerged. After a week post transfection resistant colonies were harvested by ring cloning and transferred to wells of a 24 well plate. Eleven different pBabe/PgBgp150 clones were grown up by transferring cells into sequentially larger culture vessels at each passage. After four passages the DNA was extracted from a confluent cell monolayer in a T75 flask for each clone to check for the presence of gp150 DNA by PCR. Primers PGB1 and IA4 which encompass the whole PgBgp150 cassette were used. Of the clones, six produced a clear product and a further two had faint bands at the correct size. Figure 3.2.9 shows the results of a diagnostic PCR, clone A1 produced the most intense product, clone A2 was one of two samples with a faint band, a product was not observed for clones A3 and A4.

Figure 3.2.8. Vector pBabe/PgBgp150 for the generation of a gp150 complementary cell line



The structure of pBabe/PgBgp150. The gp150 gene is located downstream of the putative gB promoter (PgB) to enable induction of gp150 expression upon infection of mammalian cells containing pBabe/PgBgp150. The vector backbone consists of pBabePuro a retroviral vector based on Moloney murine leukemia virus (Morgenstern & Land, 1990) and contains the LTR - long terminal repeat, and gag retrovirus sequences. The LTR shown to the left of the PgBgp150 insert contains a strong promoter from which some low level constitutive expression of gp150 may be directed. P_{SV40} - Simian virus 40 promoter, Puro - puromycin resistance, Amp^R - ampicillin resistance, ori - bacterial origin of replication.

Figure 3.2.9. Detection of gp150 DNA in mammalian cells transfected with pBabe/PgBgp150



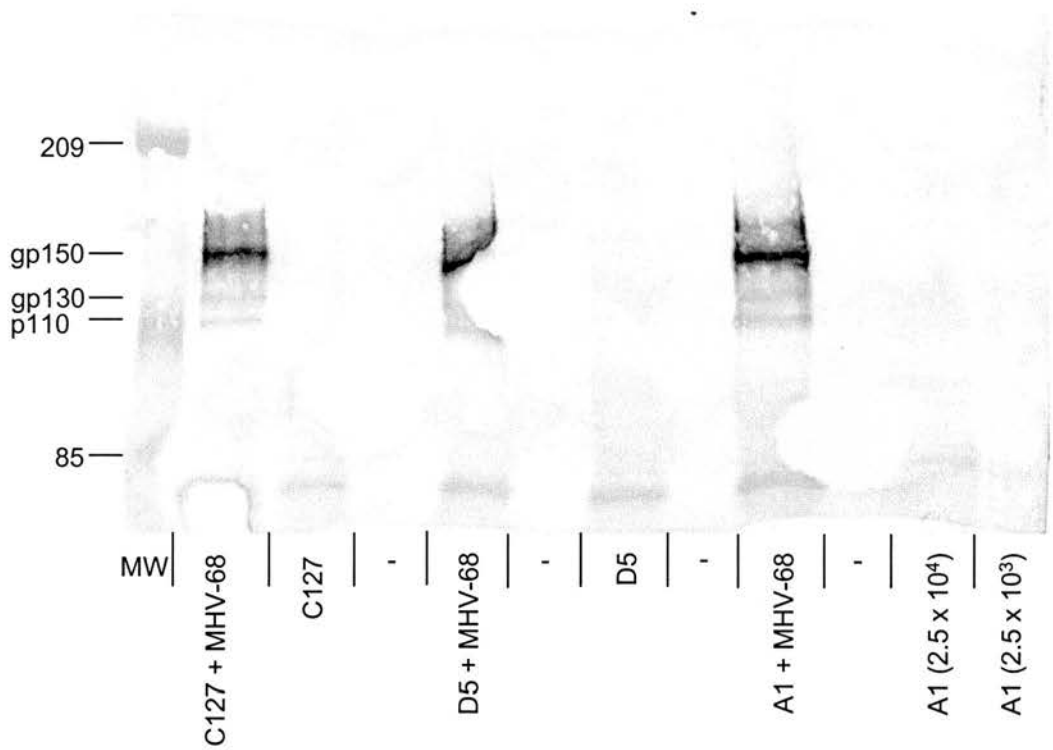
In order to test for the presence of gp150 DNA within clones of C127 cells transfected with pBabe/PgBgp150 polymerase chain reaction (PCR) analysis was performed on 100ng of DNA extracted from each cell line. Forty cycles were done using primers PGB1 and IA4, which encompass the whole PgBgp150 cassette of ~1.8kbp. Products were analysed on 0.7% agarose gels containing ethidium bromide and visualised using a UV transilluminator. Lanes: 1- 1 kb DNA ladder marker, size of pertinent bands are shown at the left, 2 - vector pPgBgp150, positive control, 3 - no DNA negative control, 4 - C127 cells, negative control, 5 & 6 - cells transfected with pBabePuro (no insert) selected using puromycin, negative control, 7,8,9 & 10 - Clones A1, A2, A3 & A4, respectively, - transfected with pBabe/PgBgp150 and selected using puromycin. Note: A faint band of ~1.8kbp could be seen by eye in lane 8 however, it is difficult to see in the photograph of the gel. The arrows indicates the position of the expected 1.8kbp product.

Although the gp150 gene had been positioned under the control of PgB the possibility existed that low level expression of gp150 may occur in the absence of virus induction of gB due to transcription occurring via the strong promoter within the LTR upstream of the PgBgp150 cassette. Cell lysates of each clone were separated by SDS-PAGE and transferred to an immobilon-P membrane which was probed for the presence of gp150 using rabbit anti-gp150 antibody followed by a swine anti-rabbit alkaline phosphatase conjugated antibody and the alkaline phosphatase substrate NBT/BCIP. In addition immunofluorescence analysis was done on acetone fixed cells using the rabbit-anti-gp150 antibody followed by a swine anti-rabbit FITC conjugated antibody. The presence of gp150, using either method, was not detected, pBabe/PgBgp150 transfected cells did not appear significantly different to the pBabePuro transfected and normal C127 cells.

Next, cell lines were infected with equal amounts of MHV-68 and the level of gp150 expression was analysed by probing western blots of cell lysates with anti-gp150 antibody. Figure 3.3.1 shows a representative analysis. In previous studies using virus induced expression the level of expression of the induced protein has been much greater in the complementary cell lines compared to control cells when infected with equal amount of virus (Forrester *et al.*, 1992). However, in this case the levels of gp150 detected in gp150 DNA positive cell lines were not significantly different to the control cells.

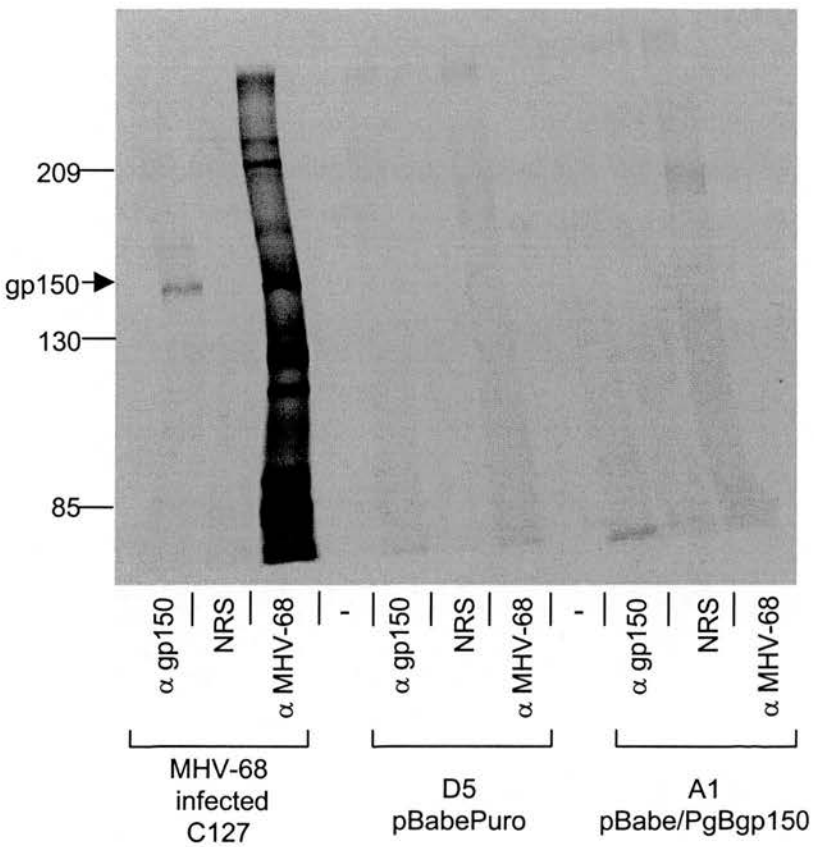
In order to increase the sensitivity of protein detection cell lines were radiolabelled using ^{35}S methionine and radioimmunoprecipitated using anti-gp150 antibody. Gp150 was successfully immunoprecipitated from MHV-68 infected C127 cells, used as a positive control, however no gp150 expression was detected in the A1 clone of pBabe/PgBgp150 transfected cells. Figure 3.3.2 shows a representative analysis of radiolabelled immunoprecipitated proteins separated by SDS-PAGE and exposed to autoradiographic film.

Figure 3.3.1. Detection of gp150 expression by SDS-PAGE and western blot analysis



Cell lysates were electrophoresed through a 5% acrylamide SDS-PAGE gel and transferred to an immunobulon P membrane. To detect the presence of gp150 the membrane was probed with rabbit anti-gp150 antiserum followed by a swine anti-rabbit alkaline phosphatase conjugated antibody. The alkaline phosphatase substrate nitro blue tetrazonium and 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) was added to visualise detected protein. Equal amounts of samples were added per well equivalent to 2.5 x 10^3 cells/well. An equal multiplicity of infection of MHV-68 (5) was used to infect cells (+ MHV-68) 18 hours prior to harvesting cells. D5 cells are C127 cells transfected with pBabePuro, A1 cells are C127 cells transfected with pBabe/PgBgp150. The positon of molecular weight markers (MW) are indicated on the left in kDa. The position of gp150 and it's precursor gp130 are indicated. P110 is derived from the use of an alternative translational initiation site in the gp150 gene (Stewart *et al.*, 1996).

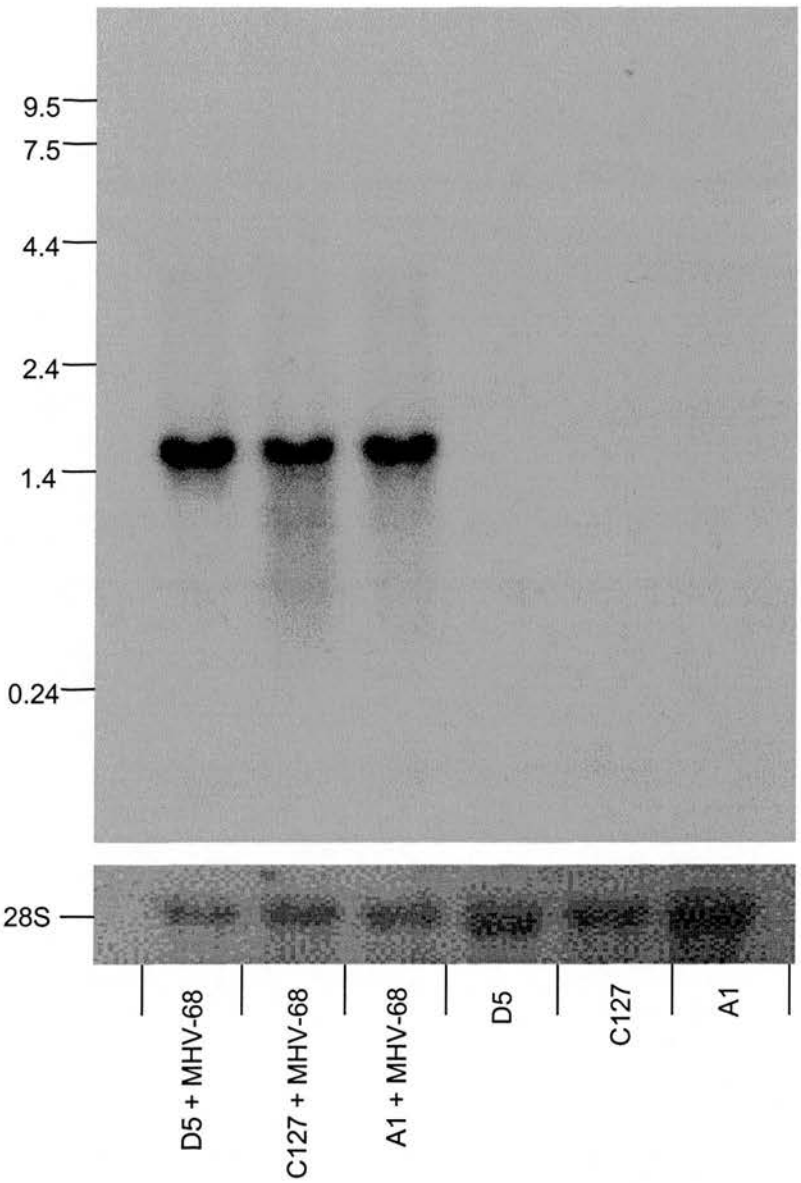
Figure 3.3.2. Immunoprecipitation analysis of gp150 expression in pBabe/PgBgp150 transfected cells



To test for expression of gp150, cells were radiolabelled with [^{35}S]-methionine. Cells were then solubilised and immunoprecipitated with rabbit anti-gp150 antiserum (α gp150), rabbit anti-MHV-68 antiserum (α MHV-68) or normal rabbit serum (NRS) using protein-A-sepharose. C127 cells were infected with MHV-68 at a multiplicity of infection (MOI) of 5, 18 hours prior to radiolabelling to be used as a positive control for immunoprecipitation of gp150. D5 is a cell line of C127 cells transfected with pBabePuro used as a gp150 negative control. The A1 cell line consists of C127 cells transfected with pBabe/PgBgp150. Immunoprecipitated proteins were electrophoresed through a 5% acrylamide SDS-PAGE gel, the gel was dried and exposed to autoradiographic film. The numbers to the left indicate the position of molecular weight standards (x10^3). The position of gp150 is indicated by the arrow. Panel shows appearance of film following a 3 hour exposure, further exposures of 24 hours up to 5 days were also done, no further significant bands were observed.

RNA extraction was done to determine if there was low level expression of gp150 mRNA in gp150 DNA positive cells and if the level of gp150 mRNA was greater in these cells following infection with MHV-68 in comparison to control cells infected with the same multiplicity of infection (MOI of 5) of MHV-68. Extracted cytoplasmic RNA was separated by agarose gel electrophoresis and transferred to a nylon membrane which was probed with a ^{32}P radiolabelled gp150 DNA probe. The DNA target used to generate the probe by random primed labelling was the entire gp150 gene, generated as a PCR product using primers IE1gp150 and IE2gp150. As figure 3.3.3 shows one mRNA transcript of approximately 1.8 kb was detected in MHV-68 infected cells 18 hours post infection however, the level of expression did not differ significantly between the pBabe/PgBgp150 cells in comparison to control cell lines. In the absence of MHV-68 infection no mRNA was detected by the gp150 specific probe in any of the cell lines.

Figure 3.3.3. Analysis of gp150 mRNA expression by cells transfected with pBabe/PgBgp150



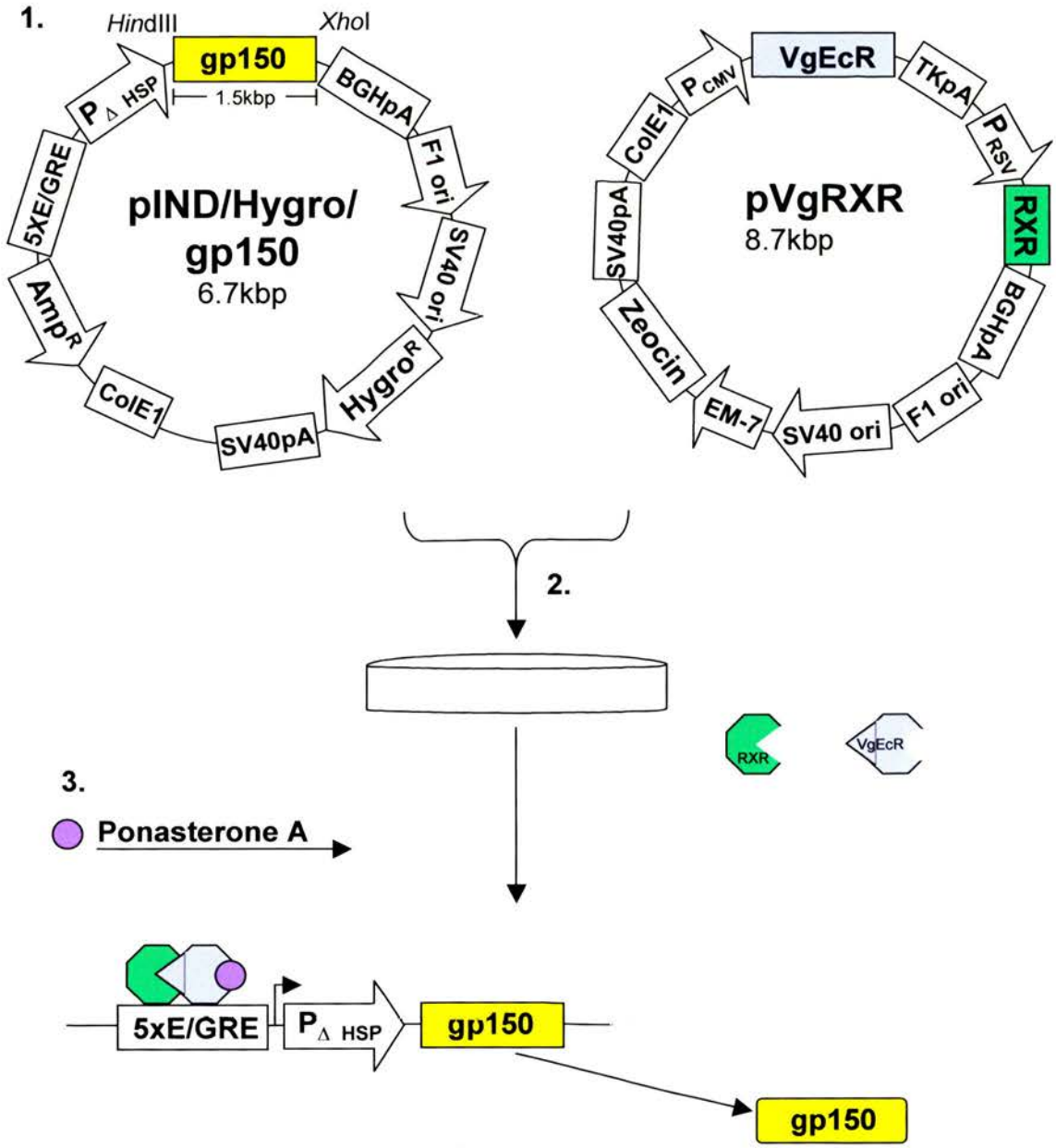
Cells were infected with MHV-68 at a multiplicity of infection (MOI) of 5 (+ MHV-68) or left uninfected. At 18 hours post infection cells were harvested and their cytoplasmic RNA extracted. Equal quantities of RNA extracted from each sample, approximately 5μg, were electrophoresed through a 1.2% agarose gel and transferred to a nylon membrane by capillary transfer. The membrane was probed with a ^{32}P radiolabelled probe generated by random primed labelling using target DNA encoding the entire gp150 gene. The membrane was then exposed to autoradiographic film for 18 hours. The main image shows an autoradiogram of the Northern blot. The numbers on the left indicate the position of RNA markers in kilobases. The lower panel shows an ethidium bromide-stained image of 28S rRNA as a control for RNA loading and integrity.

3.2.1 Ecdysone Inducible Mammalian Expression System

As an alternative method to express gp150 in mammalian cells an ecdysone inducible system for protein expression was employed. The advantage with this system is that protein expression is inducible by external control i.e. the addition of an induction agent - ecdysone or an ecdysone analogue. Inducible expression would be valuable in the event that gp150 proved toxic to cells. The ability to turn on protein expression would also provide the opportunity to perform experiments determining the susceptibility of the same cell line to MHV-68 infection in the presence and absence of gp150 expression. For example, a cell line engineered to express HSV-1 gD is resistant to HSV-1 infection. HSV-1 can attach to cells expressing gD but is not able to penetrate (Campadelli-Fiume et al., 1990) this is likely to be due to the cellular gD sequestering the cell surface molecules which HSV-1 requires to interact with via gD in order to infect the cell (Geraghty *et al.*, 2000).

Figure 3.3.4 shows an outline of the ecdysone-inducible mammalian system. The gene for expression i.e. *gp150* is cloned into an expression plasmid under the control of the minimal heat shock promoter, upstream of which is an ecdysone/glucocorticoid hybrid responsive element (E/GRE). The hybrid response element consists of one half site from the natural *Drosophila* ecdysone response element and one half-site from the glucocorticoid response element. The expression vector is co-transfected into mammalian cells with a plasmid encoding the ecdysone receptor pVgRXR. In the presence of the sterioid hormone ecdysone analogue, ponasterone A, expression of the gene of interest is activated. Activation is initiated in the presence of ponasterone A as this enables the hybrid ecdysone receptor (VgEcR/RXR) which is a heterodimer of the ecdysone receptor (VgEcR) and the mammalian retinoid X receptor (RXR) encoded by pVgRXR to bind E/GRE on the expression vector. The ecdysone receptor, derived from the natural *Drosophila* ecdysone receptor, involved in induction of insect molting, has been modified to contain the HSV VP16 transactivation domain. Upon binding of the VgEcR/RXR heterodimer to the E/GRE on the expression vector transcription of the inserted gene is activated from the minimal heat shock promoter via the VP16 transactivation domain.

Figure 3.3.4. The ecdysone inducible mammalian expression system for expression of gp150



1. In order to express gp150 in the ecdysone inducible expression system the gene encoding gp150 was ligated into vector pIND/Hygro between restriction sites *Hin*dIII and *Xho*I to form pIND/Hygro/gp150. 2. pIND/Hygro/gp150 was co-transfected with pVgRXR, using electroporation, into C127, COS or BHK cells. 3. pVgRXR encodes a heterodimer, consisting of the modified ecdysone receptor (VgEcR), derived from *Drosophila*, and the mammalian retinoid X receptor, which binds the hybrid ecdysone response element (E/GRE) in the presence of ponasterone A (a synthetic analogue of ecdysone). As the heterodimer binds to E/GRE transcription of gp150 is activated from the minimal heat shock promoter ($P_{\Delta HSP}$) via a HSV VP16 transactivation domain which the VgEcR has been modified to contain. Abbreviations: BGHpA - bovine growth hormone polyadenylation signal, f1 ori - f1 origin of replication, SV40 ori - simian virus 40 origin & promoter, SV40pA - simian virus 40 polyadenylation signal, $Hygro^R$ - hygromycin-B-phosphotransferase, ColE1 - *E. coli* origin, Amp^R - β-lactamase, P_{CMV}- CMV immediate early promoter, TKpA - thymidylate kinase polyadenylation signal, Zeocin - zeocin resistance, EM-7 - EM-7 promoter, P_{RSV} - Rous sarcoma virus promoter.

3.2.2 Introduction of *gp150* into the Ecdysone System

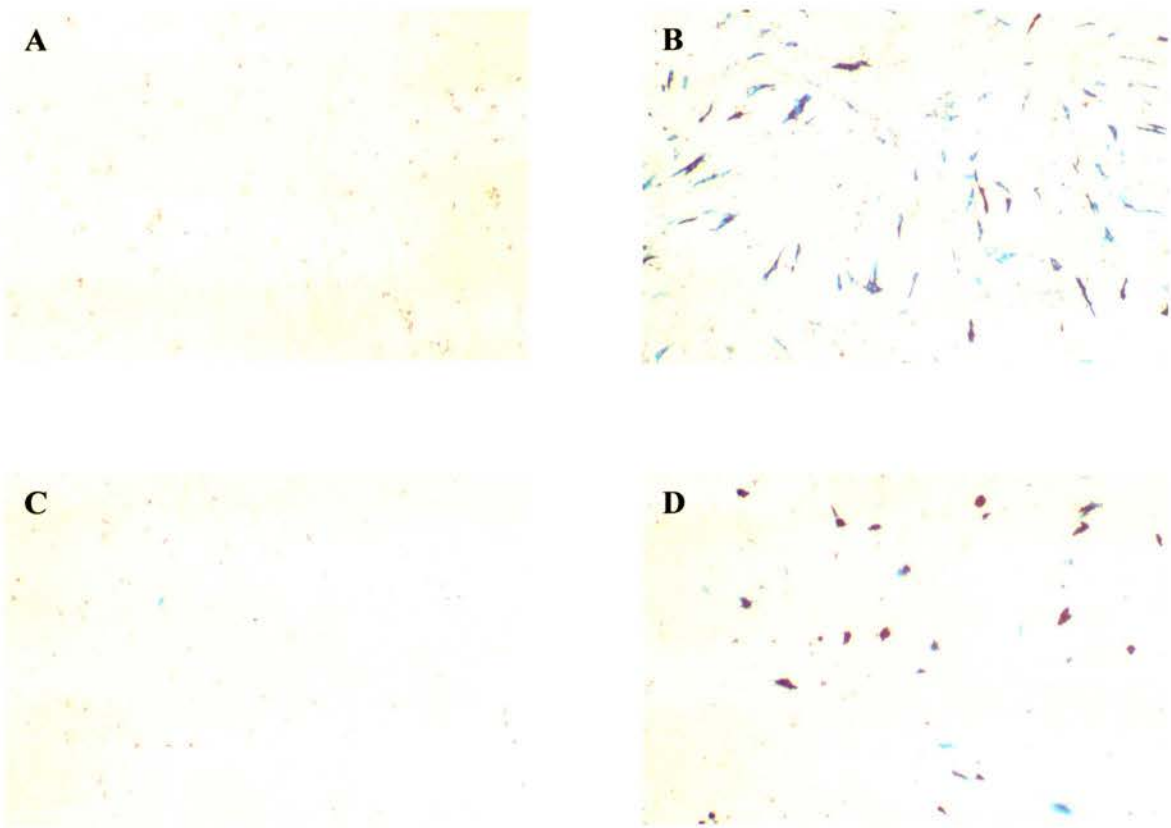
The entire gp150 gene was amplified by PCR using primers IE1gp150 and IE2gp150. In addition primers IE1gp150 and MA2stop were used to amplify the gp150 gene without the hydrophobic transmembrane domain and cytoplasmic tail encoding region. This product, when expressed from an expression vector was designed to produce secreted gp150 due to the lack of the hydrophobic membrane anchor. The expression of glycoproteins lacking their transmembrane hydrophobic region is a common method used to produce soluble glycoproteins secreted into the cell culture medium (Motz *et al.*, 1987, Madej *et al.*, 1992). Both the 1481bp product of primers IE1gp150 an IE2gp150 and the 1391bp product of IE1gp150 and MA2stop (named Xgp150) were purified and then cut with *Hin*dIII and *Xho*I. The cut products were each ligated into the expression vector pIND/Hygro prepared by digestion with *Hin*dIII and *Xho*I (refer to figure 3.3.4 for map of pIND/Hygro). Likewise, the whole gp150 gene was also ligated into pIND(SP1)/Hygro. The expression vector pIND(SP1)/Hygro is a modified version of pIND/Hygro differing in that it contains three binding sites for the transcription factor SP1 positioned between the E/GRE and minimal heat shock promoter designed to increase absolute expression levels fivefold over those obtained with pIND/Hygro (Kadonaga *et al.*, 1987, No *et al.*, 1996).

XL-1 blue *E. coli* were transformed with the ligation mixes using the heat shock method. Transformants were selected using ampicillin, 16, 17 and 2 colonies grew for the pIND/Hygro plus gp150, pIND(SP1)/Hygro plus gp150 and pIND/Hygro plus Xgp150 ligation mixes, respectively. Plasmid DNA was extracted from liquid cultures of these clones and then digested using *Hin*dIII and *Xho*I. All colonies for each transformation contained plasmids with the insert. For each construct i.e. pIND/Hygro/gp150, pIND(SP1)/Hygro/gp150 and pIND/Hygro/Xgp150 a large scale plasmid preparation was made using the CsCl method. In addition, large scale DNA preparations were made of pVgRXR and pIND/Hygro/*lac*Z. The vector pIND/Hygro/*lacZ* contains the β-galactosidase gene and was used as a positive control for mammalian transfection and expression.

Transfection of each expression construct in conjunction with an equal amount (30μg) of pVgRXR into BHK cells was done using electroporation. The induction agent ponasterone A (5μM) was added to cultures 24 hours post transfection. Cells were harvested 22 hours post induction and analysed for expression of gp150 using immunofluorescence and western blotting. Using anti-gp150 antibody to probe western blots of cell lysates and in immunofluorescence analysis on both acetone or paraformaldehyde fixed cells did not result in detection of gp150. The supernatant of cells transfected with pIND/Hygro/Xgp150 + pVgRXR was retained for analysis by western blotting however no gp150 was detected. In contrast, expression of β-galactosidase was detected in cells co-transfected with pIND/Hygro/*lacZ* and pVgRXR as shown in figure 3.3.5. This confirmed that the large DNA preparation of pVgRXR and the induction agent were functional. Repeat transfections were performed however gp150 was not detected. Transfection of COS and C127 cells was also done. Analysis by immunofluorescence and western blotting did not result in the detection of gp150. Increasing the concentration of the induction agent ponasterone A to 10μM did not alter the outcome. Sequence analysis of the gp150 plasmid constructs using the gp150 spanning primers and the ecdysone forward and BGH reverse primers showed the gp150 inserts to be 100% intact.

In addition to transient transfection analysis selection of COS and C127 cell lines containing pVgRXR and each expression construct was done using zeocin and hygromycin. Prior to this the sensitivity of these parent cell lines to the selective agents was established in order to determine the appropriate antibiotic concentration that inhibits cell growth to use for selection of transformed cells. By monitoring growth of cell lines in a range of concentrations (e.g. 0, 50, 125, 250, 500, 750 and 1000μg/ml) of each drug the minimum concentration of drug that inhibited cell growth was determined to be 150μg/ml for both zeocin and hygromycin. After a week post transfection of cells under these selection agents colonies of cells grew out – approximately 5 per well of a six well plate. Clones were isolated by ring cloning and grown up for induction and analysis of expression as before. No gp150 expression was detected. Presence of gp150 DNA was confirmed by PCR using primers MA1 and MA2. All clones, which were maintained under antibiotic

Figure 3.3.5. Analysis of protein expression (β-galactosidase) using the ecdysone inducible system



To test the transfection efficiency and demonstrate β-galactosidase protein expression using the ecdysone inducible system BHK cells (10^6) were transfected using electroporation with various plasmids (30μg each). Twenty four hours post transfection with pIND/Hygro/lacZ + pVgRXR 5μM ponasterone A was added to induce expression. Forty eight hours post transfection cells expressing β-galactosidase were stained blue by the application of the substrate X-gal. Cells stained for β-galactosidase expression are shown - panel A: normal BHK cells, untransfected B: BHK cells transfected with pMV10; constitutive β-galactosidase expression, with *lacZ* under the control of the CMV IE-1 promoter, C & D: BHK cells co-transfected with the ecdysone receptor plasmid -pVgRXR and β-galactosidase expression plasmid pIND/Hygro/*lacZ*, C - uninduced, D - 24 hours post induction; addition of ponasterone A (an analogue of ecdysone). Original magnification: 50x.

selection throughout, grew very slowly (approximately 82 times slower) in comparison to standard COS cells.

BINDING & ANTISERA STUDIES

3.2.3 Generation of Gp150-His

To generate purified gp150 for functional studies a gp150-His fusion protein was made. The expression of the extracellular portion of gp150 as a recombinant protein fused to a hexahistidine tag provided an amenable method to purify the protein on nickel charged resin. The extracellular domain of gp150 as opposed to the whole protein was selected as this was likely to facilitate any cellular binding or entry role the protein may have and be more soluble in the absence of the hydrophobic transmembrane domain. In addition the hexahistidine tag made up a relatively small proportion of the protein and was therefore considered less likely to interfere with the structure and function of gp150 in comparison to other possible expression systems that employ the attachment of larger protein molecules e.g. glutathione-S-transferase to facilitate purification of the protein.

Bacterial expression of the gp150 recombinant protein was used because of the ease of manipulation and the ability to grow bacteria rapidly in bulk culture. Although bacterial proteins do not undergo the same post translational modifications as those of eukaryotic cells, bacterial expression was considered a worthwhile approach as gp150 has very limited glycosylation. Studies indicate that only one of the three potential N-linked glycosylation sites are occupied (Stewart *et al.*, 1996). Investigations show that other viral glycoproteins lacking their oligosaccharide modifications retain their function in binding and fusion. For example, gD of HSV remains biologically active in the absence of N-linked glycosylation (Sodora *et al.*, 1991a & b).

Cloning Gp150-His

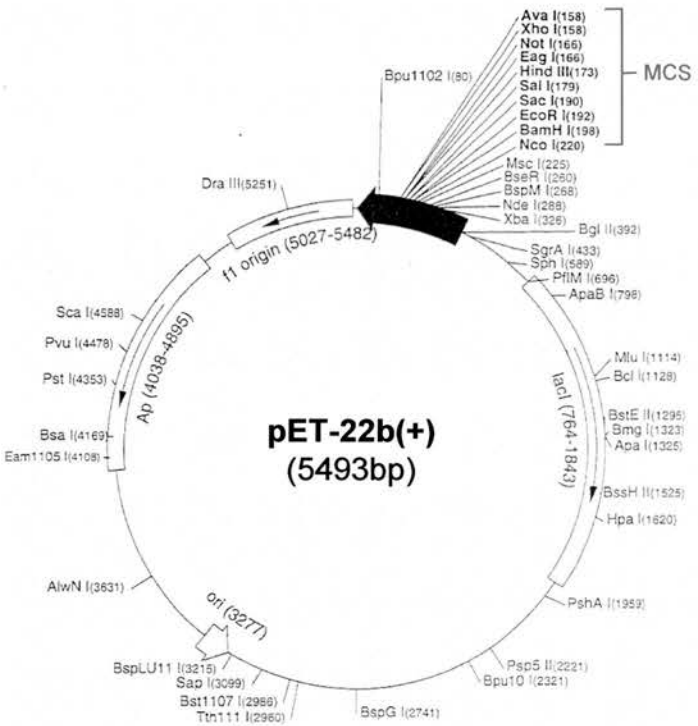
A 1.3kbp DNA sequence, encoding the extracellular domain of gp150 (i.e. the region between the signal peptide sequence and the transmembrane domain, see figure 1.8) was cloned from pSP72/gp150 (this vector contains the whole of the gp150 gene) by PCR using primers MA1 and MA2 (for primers and conditions see section 2.1.8). The resulting product was purified and the ends digested using *Nco*I and *Xho*I. The cut DNA was then purified and ligated into the plasmid pET-22b upstream of and in frame with a hexahistidine encoding region and downstream of a periplasmic leader sequence (*pel* B) (see figures 3.3.6a, b & c). The periplasmic leader sequence directs the fusion protein into the periplasm of the host bacteria and is cleaved on entry. Figures 3.3.6a & b depict the pET-22b plasmid, the region upstream of the multiple cloning site contains the T7 promoter which can be activated by T7 RNA polymerase. For expression of the gene the presence of T7 RNA polymerase is required therefore bacterial hosts that encode a chromosomal copy of the T7 RNA polymerase gene are used such as BL21(DE3) selected in this case. The bacterial strain BL21(DE3) is a lysogen of the bacteriophage DE3 from which a DNA fragment encoding the *lacUV5* promoter and the gene for T7 RNA polymerase is inserted into the bacterial chromosome (Studier & Moffatt, 1986). The only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG).

Prior to ligation pET-22b was cut with *Nco*I and *Xho*I and then treated with calf alkaline phosphatase to prevent self ligation of the plasmid. Competent *E.coli* were transformed with the ligation mix using the heat shock method. Transformed bacteria were selected using ampicillin. From the 400 colonies that grew, 12 were picked, grown up and minipreped. The resultant plasmids were cut with *Nco*I and *Xho*I . Three of the 12 plasmids produced two bands of approximately 5.5kbp and 1.3kbp, indicating correct insertion of gp150. These plasmids were named pET-22b/gp150. Plasmids from three clones were used to transform BL21(DE3) bacteria by the colony transformation method. Successfully transformed bacteria were selected for using carbenicillin. Of over 250 colonies that grew for each

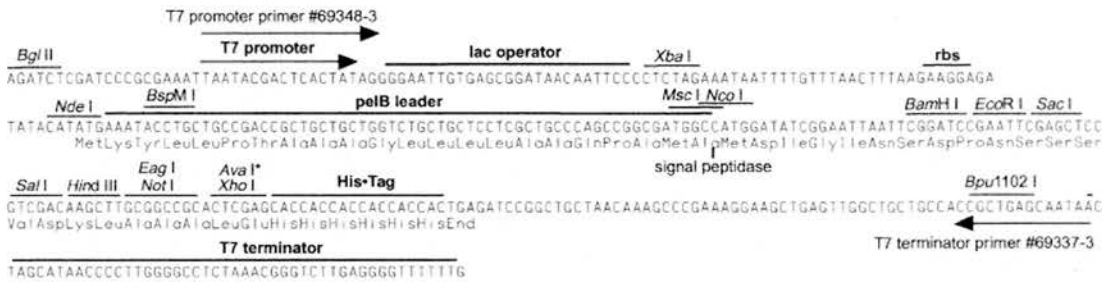
Figure 3.3.6a & b. Expression vector pET-22b used for the expression of hexahistidine tagged fusion proteins

A.

pET-22b sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning site	158-225
Histidine coding sequence	140-127
T7 terminantor	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
B-lactamase (Ap)	4038-4895
F1 origin	5027-5482

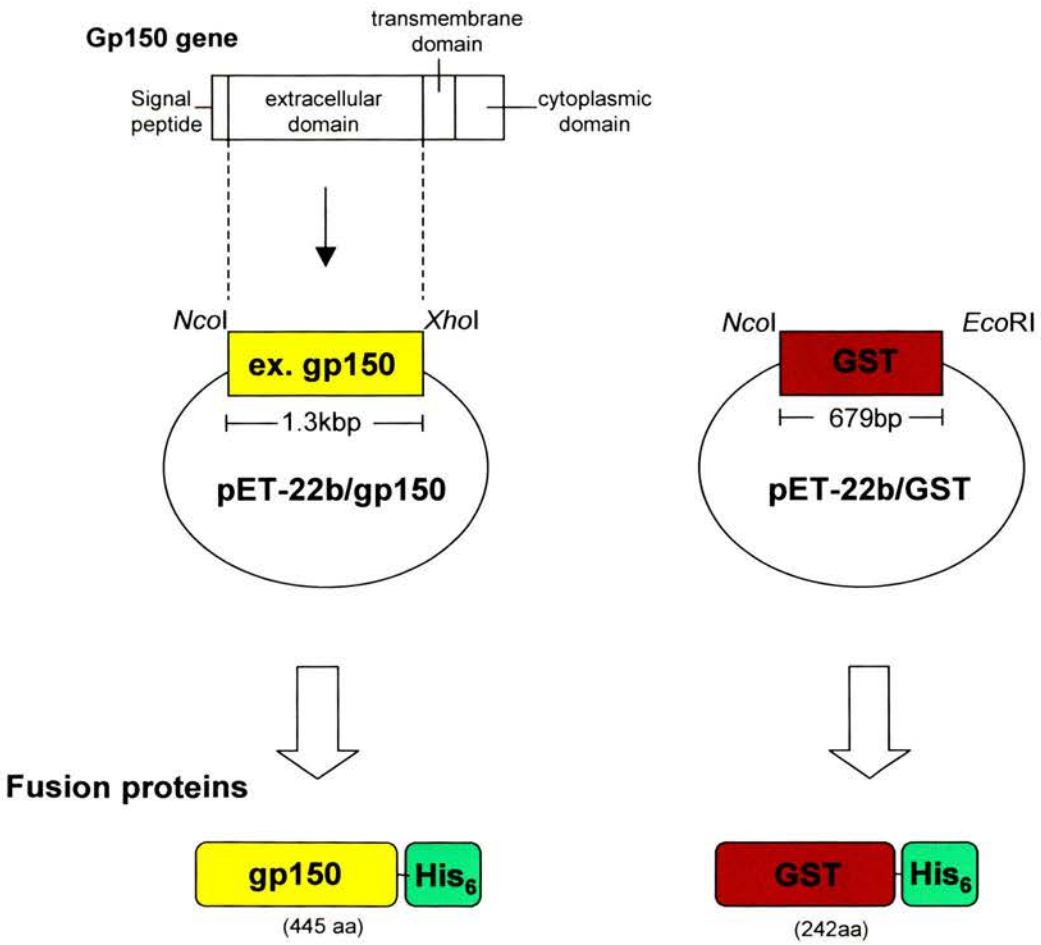


B. pET-22b cloning/expression region



A. The region upstream of the multiple cloning site (MCS) contains the T7 promoter which can be activated by T7 RNA polymerase. For expression of the gene inserted in the MCS the presence of T7 RNA polymerase is required therefore bacterial hosts that encode a chromosomal copy of the T7 RNA polymerase gene are used for example *E.coli* strain BL21(DE3). The bacterial strain BL21(DE3) is a lysogen of the bacteriophage DE3 from which a DNA fragment encoding the *lacUV5* promoter and the gene for T7 RNA polymerase is inserted into the bacterial chromosome (Studier & Moffat, 1986). *lacUV5* is a promoter inducible by isopropyl-β-D-thiogalactopyranoside (IPTG) and is known to direct transcription of the T7 RNA polymerase gene which in turn induces expression of the recombinant fusion protein. **B**. Cloning region of pET-22b. For the generation of gp150-His and GST-His fusion proteins the extracellular domain of *gp150* was inserted into pET22b between the *Nco*I and *Xho*I restriction sites, the glutathione-S-transferase (GST) gene was inserted between the *Nco*I and *Eco*RI restriction sites, respectively. The fusion proteins have a pelB leader sequence which directs the fusion proteins into the periplasm of the host bacteria, upon entry of the periplasmthe pelB leader domain is cleaved from the proteins.

Figure 3.3.6c. pET-22b based constructs for the expression of gp150-His and GST-His fusion proteins



To generate gp150 and glutathione-S-transferase (GST) hexahistidine tagged fusion proteins expression vector pET-22b was used. The extracellular encoding domain of *gp150* was ligated into the multiple cloning site (MCS) of pET-22b between the *Nco*I and *Xho*I restriction sites. The GST gene was ligated into the MCS between *Nco*I and *Eco*RI. The number of amino acids of each fusion protein, following cleavage of the pelB leader signal, is indicated in brackets.

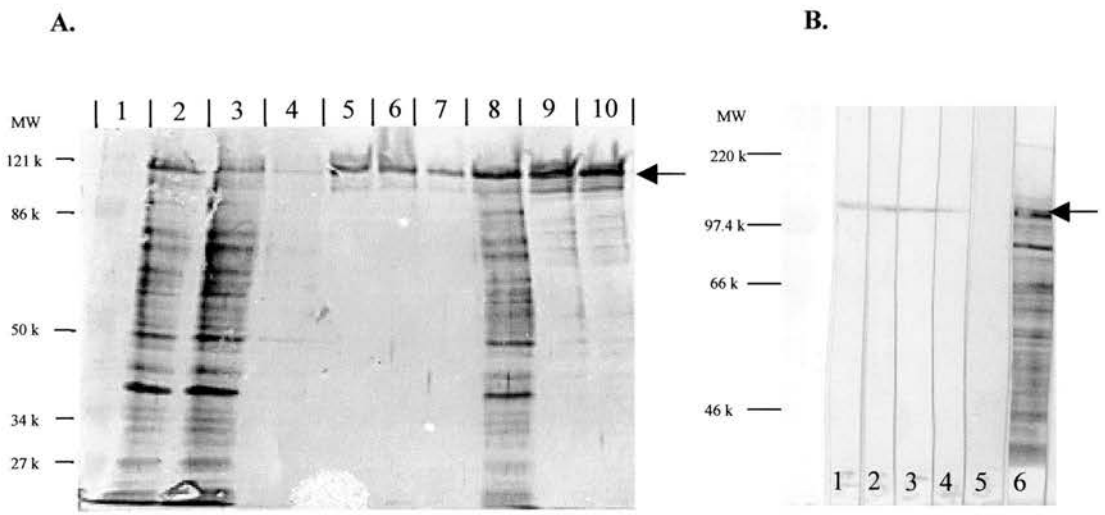
pET-22b/gp150 used one colony was picked. The presence of the chosen plasmids was checked by digestion of minipreps of these clones as before.

Expression & Purification of Gp150-His

By sequence analysis the gp150-His fusion protein was predicted to have a molecular weight of 45,515 and an isoelectric point of 3.84. Clones containing pET-22b/gp150 were grown up in liquid culture using carbenicillin and induced to express the fusion protein by addition of IPTG. To test for production of fusion protein the induced bacteria were harvested three hours post induction, sonicated and analysed by SDS-PAGE. A predominant band at 120 kDa was present for each of the transformants. Subsequently, expression of the fusion protein in the periplasmic fraction was confirmed by SDS-PAGE analysis of the periplasmic fraction of the induced bacteria. Samples taken at increasing time points post induction of expression were analysed by SDS-PAGE and an induction time of 2.5 hours was determined to be the optimum time for protein production. One transformant was selected and induced to express the fusion protein followed by small scale purification from the periplasmic fraction using Ni^{2+} charged resin beads (100µl settled resin). The resultant purified protein with an apparent molecular weight of 120 kDa was detected by gp150 specific antiserum on a western blot, a faint band of approximately 110kDa was also detected. In addition, these bands were detected using anti-MHV-68 antiserum. The apparent size of the gp150-His fusion protein detected (120kDa) was much greater than the predicted MW of 45,500 and this is thought to be due to the high proline content (23%) of gp150 which is known to increase the apparent MW of proline rich proteins in SDS-PAGE gels (Stewart *et al.*, 1996).

Upon confirmation that it was possible to purify gp150-His, using the Ni^{2+} charged resin, production of the protein was scaled up to 100ml and 500ml cultures, purification was monitored by SDS-PAGE and western blot analysis (see figure 3.3.7a for purification steps and concentrated protein). Detection of the 120kDa fusion protein using an anti-hexahistidine monoclonal antibody was also possible (see figure 3.3.7b).

Figure 3.3.7. Expression, purification & detection of gp150-His from bacteria carrying vector pET-22b/gp150



A. A western blot of purified gp150-His fusion protein and fractions generated during the affinity purification process using Ni^{2+}charged resin beads. The blot was probed for gp150-His fusion protein with rabbit anti-gp150 antibody followed by a swine anti-rabbit alkaline phosphatase conjugated secondary antibody. Lane: 1- molecular weight (MW) marker, 2- periplasmic fraction (PF) of gp150-His expressing bacteria, 3- PF in binding buffer prior to application to beads, 4- binding buffer wash, 5- wash buffer, 6- eluate, 7- Ni^{2+}resin beads, 8- pooled waste from each purification step, 9 & 10 - concentrated eluate (purified gp150-His). Samples were separated on a 7.5% acrylamide SDS-PAGE gel. **B.** Gp150-His detected on a western blot of the periplasmic fraction of induced bacteria probed with a range of dilutions of a mouse anti-hexahistidine monoclonal antibody (1/100, 1/500, 1/1000, 1/2000 lanes1-4 respectively) followed by a goat anti-mouse alkaline phophatase conjugated secondary antibody (lane 5 - secondary antibody alone, lane 6 - probed with anti-gp150 antibody as for blot A). Proteins were separated on a 10% acrylamide SDS-PAGE gel. The arrow indicates the position of gp150-His.

The smaller band 110kDa was not detected by the anti-hexahistidine monoclonal antibody and is likely to be a C-terminus breakdown product of gp150-His.

A sample of purified fusion protein was provided for analysis to SmithKline Beecham Biologicals as part of a collaborative effort. Amino acid analysis performed by them indicated that the protein preparation was highly pure. Using the concentration determined by this analysis and the absorbance of the sample at 280nm measured by spectrophotometry it was possible to calculate, using Beer-Lamberts law (refer to section 2.4.6), the molar extinction coefficient of the gp150-His protein which was determined to be 70,000 $M^{-1}cm^{-1}$.

From 100ml culture 0.093 – 0.17mg of gp150-His protein could be purified, approximately 1.3mg per litre culture. Western blot analysis indicated that some protein remained on the beads following one elution step. In order to improve protein yield two separate elution steps were carried out. To limit protein eluting from the beads during the wash step the level of imidazole in the wash buffer was reduced from 60mM to 20mM. Purification of protein from a litre culture was done however purification from smaller volumes (100ml and 500ml) proved more efficient. Increasing the volume of Ni^{2+} resin used did not enhance the amount of protein purified indicating that the protein yield was predominantly restricted by the level of protein expressed by the bacteria.

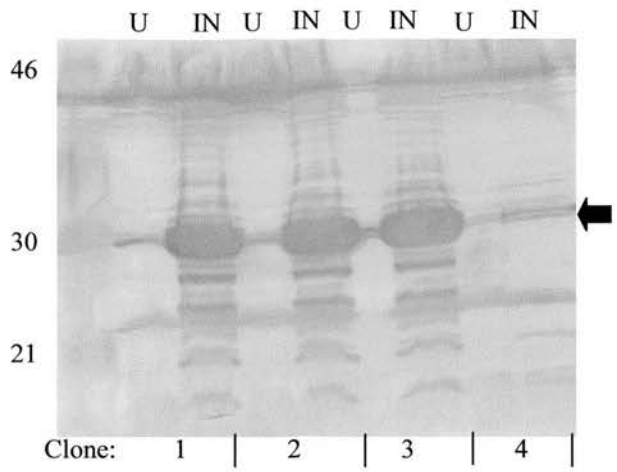
3.2.4 Generation of GST-His – Using Vector pET22b

In order to provide a control for the gp150-His fusion protein a GST-His recombinant fusion protein was generated. This consisted of the carboxy terminus of the glutathione-S-transferase gene encoding a 27kDa protein attached to a hexahistidine tag. GST is a common eukaryotic cytoplasmic protein in this case derived from the parasite *Schistosoma japonicum* it belongs to a family of isozymes that detoxify a variety of xenobiotics by catalysing the interaction of reduced glutathione with these molecules (Smith *et al.*, 1986, Smith & Johnson, 1988). This was considered a feasible control protein because GST is known to be soluble in solution and the serum available to gp150 was generated against a GST-gp150 fusion protein and therefore could be used to identify GST-His. GST has been used as a

control protein in other binding studies and was unlikely to display cell surface specific binding (Xuan *et al.*, 1995, Kitay *et al.*, 1996).

The gene encoding GST was amplified from the plasmid pGEX-2T by PCR using primers GST1n and GST2e (see section 2.1.8 for primers and conditions). The resulting product of 689 bp was purified and then cut with *Nco*I and *Eco*RI. The cut DNA was purified and ligated into the plasmid pET-22b upstream of and in frame with a hexahistidine encoding region and downstream of a periplasmic leader sequence (*pel* B) (see figures 3.3.6a, b & c). Prior to ligation pET-22b had been prepared by digestion with *Nco*I and *Eco*RI followed by dephosphorylation to prevent self ligation. Competent *E. coli* were transformed with the ligation mix using the heat shock method. Transformed bacteria were selected using carbenicillin. From the 45 colonies that grew, 12 were picked, grown up and their plasmid DNA extracted. The resultant plasmids were cut with *Nco*I and *Eco*RI. All of the plasmids produced the correct banding pattern, two bands of approximately 5.5kbp and 700bp, indicating that GST had been successfully ligated into pET-22b. These plasmids were named pET-22b/GST. Four pET-22b/GST plasmid isolates were used to transform BL21(DE3) bacteria by the colony transformation method. Successfully transformed bacteria were selected for using carbenicillin. Approximately 30 colonies grew for each pET-22b/GST clone, one colony was picked from each. These colonies were grown up in liquid culture using carbenicillin and induced to express the fusion protein by the addition of IPTG. To test for production of fusion protein the induced bacteria were sonicated and analysed by SDS-PAGE. Three out of the four clones, following induction expressed a protein which resolved at approximately 27kDa by SDS-PAGE (see figure 3.3.8). To check for the presence of the histidine tag a western blot of an SDS-PAGE gel of the samples was probed with an anti-hexahistidine monoclonal antibody and the approximately 27kDa band was detected.

Figure 3.3.8. Expression of GST-His from BL21(DE3) bacteria transformed with vector pET-22b/GST

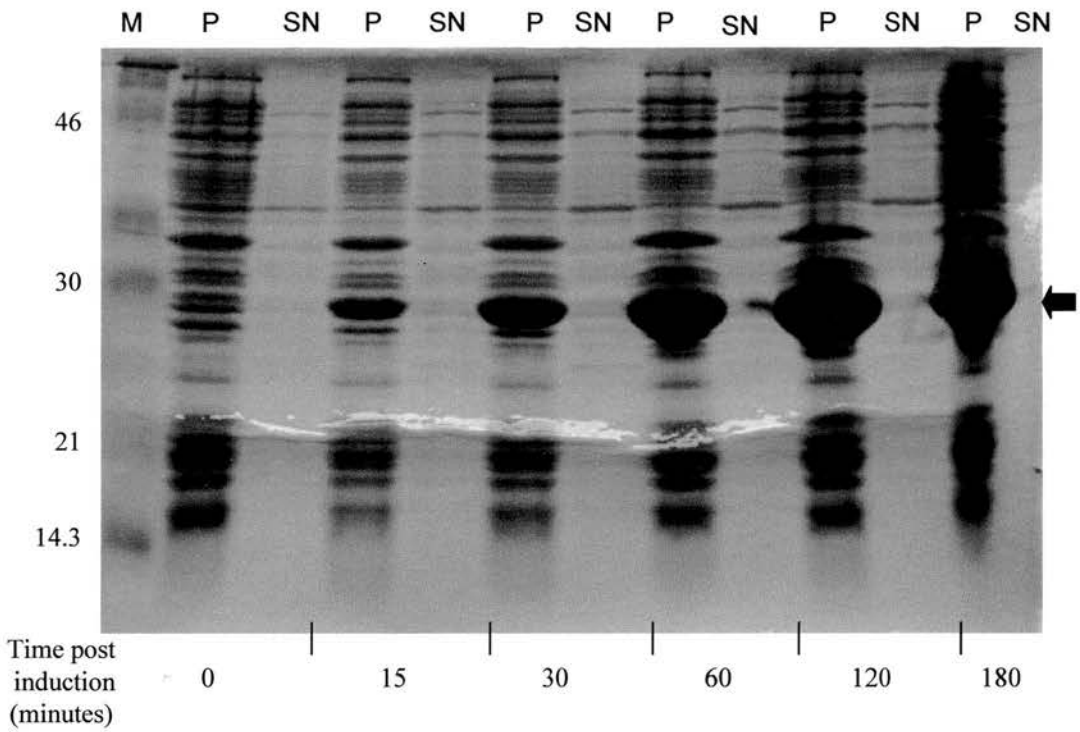


Western blot of bacteria transformed with pET-22b/GST induced to express the fusion protein GST-His by addition of isopropyl- β-D-thiogalactopyranoside (IPTG). Samples of bacterial culture prior to induction (U) and 3 hours post induction (IN) were sonicated and the whole bacterial lysates separated on a 14% acrylamide SDS-PAGE gel followed by western blotting. The western blot was probed with rabbit anti-gp150 antibody followed by a swine anti-rabbit alkaline phosphatase conjugated secondary antibody. The arrow indicates the location of the GST-His fusion protein. Numbers at the left hand side indicate molecular weight in kDa.

Unfortunately, the GST-His protein could not be detected in the purified periplasmic fraction of induced bacteria. The GST-His protein for each clone appeared insoluble, detectable in the pellet but not the supernatant of sonicated bacteria. The lack of detectable protein in the periplasmic fraction may have been due to the *pel* B leader signal undergoing mutation or the protein being translated from the GST AUG rather than the *pel* B AUG. Alternatively, the GST-His may have been expressed at such high levels that it formed aggregates and inclusion bodies and therefore was not present in the periplasmic fraction. As it may have been possible that at early times post induction there was more soluble GST-His and as it accumulated the protein became insoluble and formed inclusion bodies a time course was carried out. Following induction of bacteria, samples were harvested at increasing time intervals post induction (15, 30, 60, 120 and 180 minutes) and the levels of soluble and insoluble fusion protein assessed. Analysis of samples by SDS-PAGE showed the majority of induced fusion protein to be insoluble from initial expression onwards (see figure 3.3.9).

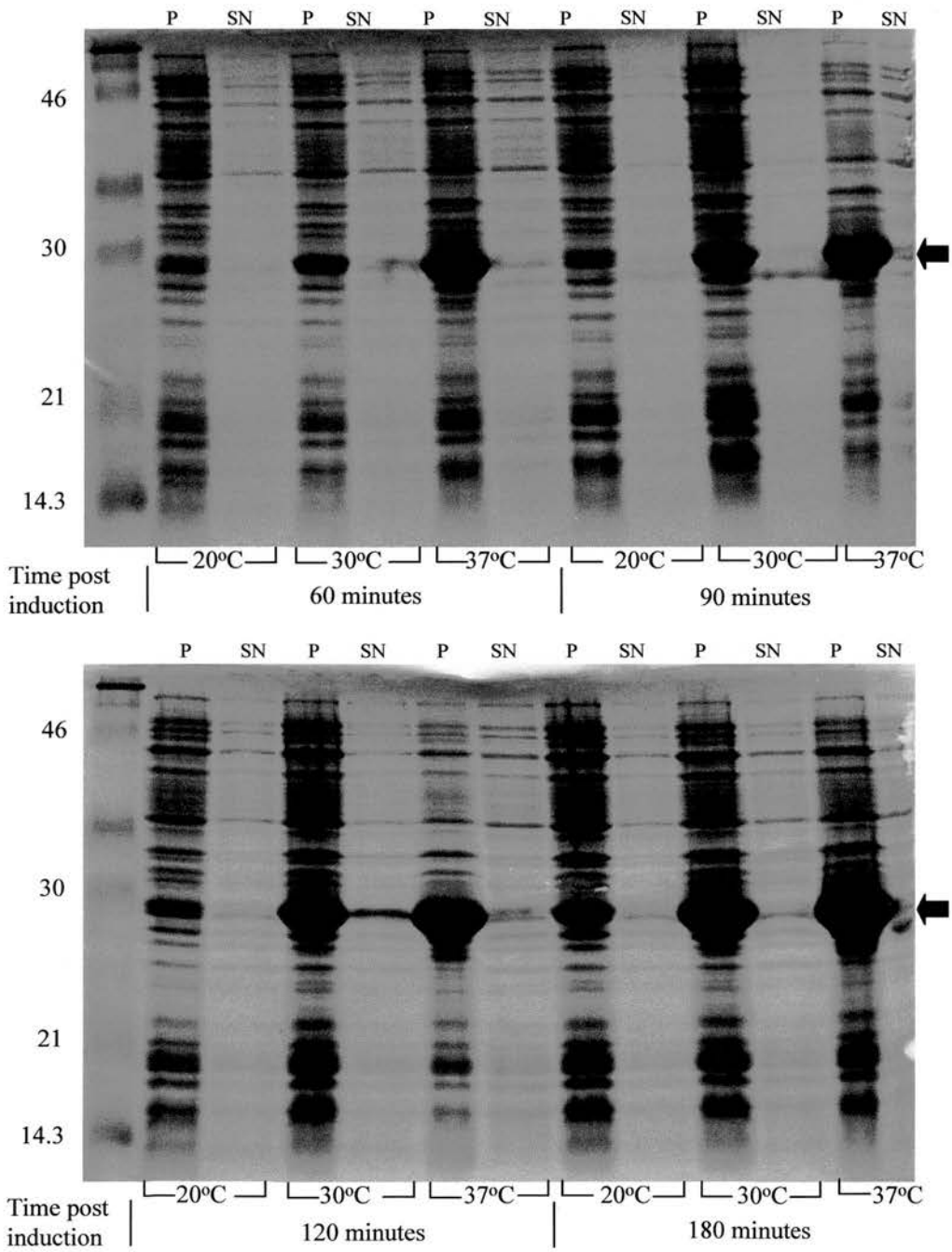
To investigate if expression at lower temperatures might enhance soluble protein production cultures for each clone were grown and induced at three different temperatures (20°C, 30°C and 37°C). Samples were collected at 60, 90, 120 and 180 minutes post induction, sonicated and the pellet and supernatant analysed by SDS-PAGE. With increasing temperature higher levels of protein were expressed for each time point post induction, the majority of protein was insoluble. At 2 hours post induction, bacteria grown at 30°C did appear to have more GST-His in the soluble fraction (see figure 3.4.1) therefore an attempt was made to purify GST-His, using Ni^{2+} charged resin, from the supernatant of whole bacteria lysate following production in these conditions. Unfortunately the purification was unsuccessful, no significant band of purified protein was present in the elute buffer, and only a very faint band when concentrated by centrifugal filtration could be seen by SDS-PAGE analysis.

Figure 3.3.9. Time course of expression of GST-His from bacteria transformed with pET-22b/GST



BL21(DE3) bacteria containing the plasmid pET-22b/GST were induced by the addition of isopropy-ß-D-thiogalactopyranoside (IPTG) to express a GST-His recombinant fusion protein. Lysates of bacteria harvested at the times indicated post induction were subject to centrifugation, the pellet/insoluble fraction (P) and supernatant/soluble fraction (SN) of each sample were separated in a 14% acrylamide SDS-PAGE gel. The SDS-PAGE gel was stained with Coomasie blue dye to visualise the proteins. The arrow indicates the position of GST-His. The numbers on the left indicate molecular weight in kDa.

Figure 3.4.1. Time course of GST-His expression from pET-22b/GST transformed bacteria at three different temperatures



BL21(DE3) bacteria containing pET-22b/GST-His were grown at three different temperatures as indicated. Samples were taken at increasing time intervals post addition of isopropy-β-D-thiogalactopyranoside (IPTG), for induction of fusion protein expression, and subject to sonication. The bacterial lysates were centrifuged and the pellet/insoluble fraction (P) and supernatant/soluble fraction (SN) were separated on 14% acrylamide SDS-PAGE gels as shown. The SDS-PAGE gels were stained with Coomasie blue to visualise the proteins. The numbers on the left hand side indicate the molecular weight in kDa. The arrow indicates the position of GST-His.

3.2.5 Generation of GST-His – Using Vector pGEX-2T

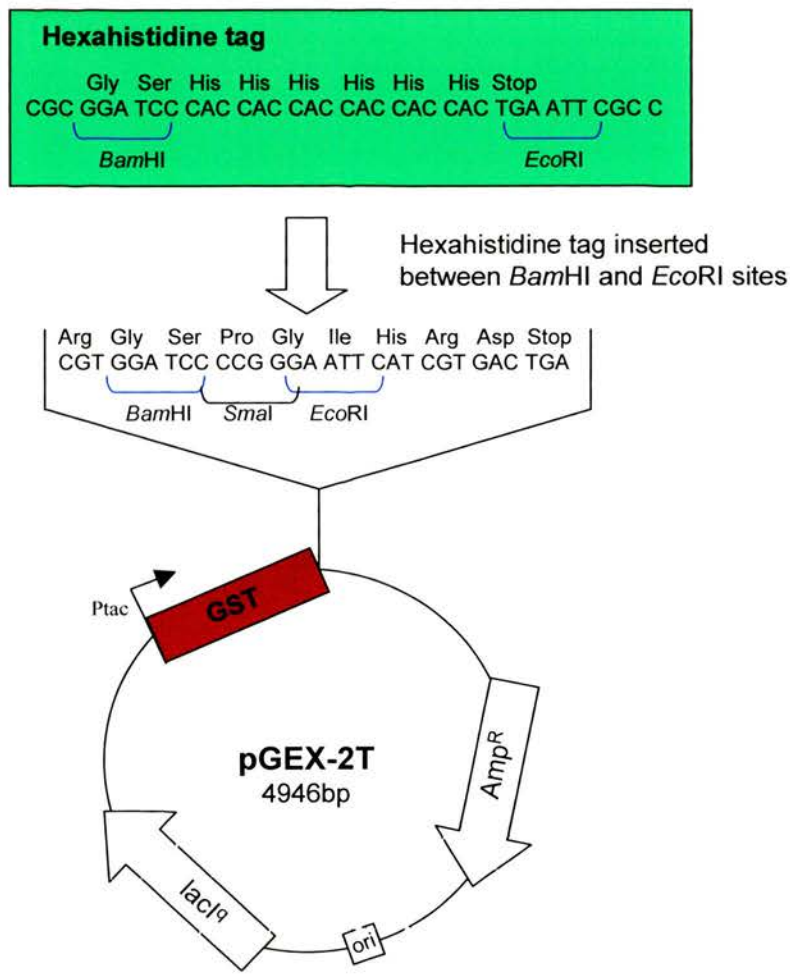
It was considered possible that the insolubility of GST-His expressed by bacteria containing pET-22b/GST may be due to the system used for expression rather than the GST-His protein structure itself therefore an alternative approach was adopted to generate a GST-His fusion protein. A hexahistidine tag was cloned downstream of the GST encoding gene in the pGEX-2T expression vector (see figure 3.4.2). This was predicted to generate a protein of 232 amino acid residues with a molecular weight of 27,133.

Sense and antisense DNA oligomers (His1 and His2 refer to section 2.1.8) were designed that encoded a hexahistidine sequence flanked by restriction sites *Bam*HI and *Eco*RI. Equimolar concentrations of the oligomers were mixed together at room temperature and then subject to digestion with *Eco*RI and *Bam*HI. The cut product was phenol/chloroform extracted and precipitated at –70°C in the presence of 0.01M $MgCl_2$ which aids the precipitation of small (<100bp) DNA fragments. The DNA fragment encoding the hexahistidine tag was then ligated into pGEX-2T (previously cut with *Eco*RI and *Bam*HI and treated with phosphatase) downstream of and in frame with the GST protein encoding gene. The ligation mix was used to transform competent *E. coli* by the heat shock method. Transformants were selected using ampicillin, only three colonies grew. These colonies were picked, grown up in liquid culture and their plasmid DNA extracted. The resultant plasmids were cut with *Sma*I alone and with *Bam*HI and *Eco*RI simultaneously. Successful insertion of the fragment should eliminate the *Sma*I site. The *Sma*I site appeared to be deleted and the correct banding pattern following *Bam*HI and *Eco*RI digestion (one band of approximately 5 kbp and another of approximately 30bp) was produced by each of the three clones however it was difficult to visualise the 30kbp fragment being so small.

Expression of GST-His

Cultures of each clone carrying pGEX-2T/His were induced for protein expression, the lysates analysed by SDS-PAGE and western blotting. A protein of approximately 27 kDa for each clone was detected on a western blot using an anti-

Figure 3.4.2. Cloning strategy for generation of GST-His fusion protein using vector pGEX-2T



The vector pGEX-2T encodes the gene for glutathione-S-transferase (GST). A DNA sequence encoding a hexahistidine tag was inserted into pGEX-2T between the *Bam*HI and *Eco*RI restriction sites to generate pGEX-2T/His. In bacteria carrying pGEX-2T/His the lac repressor (product of the $lacI^q$ gene) binds to the P_{tac} promoter repressing the induction of GST fusion protein. Upon induction with isopropyl β-D-thiogalactopyranoside derepression occurs and the GST-His fusion protein is expressed. Amp^R - ampicillin resistance gene, ori - origin of replication.

hexahistidine monoclonal antibody confirming the presence of the hexahistidine tag (see figure 3.4.3a) The protein was present in the soluble fraction of the bacterial cell lysate and was successfully purified from bacteria 150 minutes post induction of expression using the Ni^{2+} charged resin method (see figure 3.4.3b). The protein was also identified on western blots using the available anti-gp150 serum which was generated against a GST-gp150 fusion protein.

From a 500ml culture of pGEX-2T/His containing bacteria it was possible to purify 0.35 – 0.665 mg of GST-His protein, approximately 1mg/litre culture. Yields of GST-His fusion protein were calculated from the absorbance at 280nm using the relation 1 A_{280} = 0.5 mg/ml (Smith & Johnson, 1988).

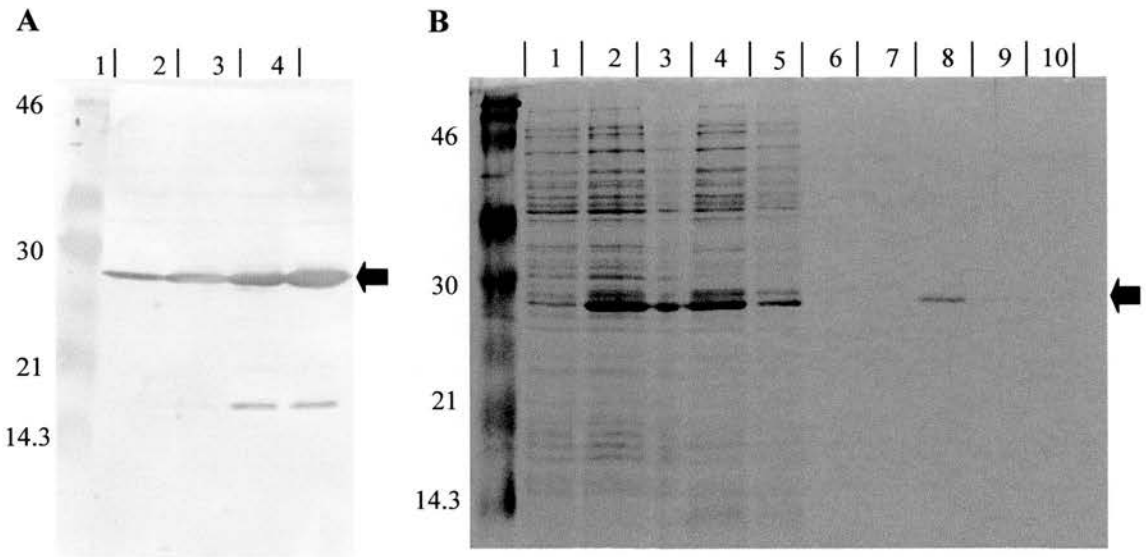
3.2.6 BINDING STUDIES

As described in section 1.4.5 virus glycoproteins can have many different functions including binding to cell surface molecules to facilitate virion attachment to cells. Futhermore, virus glycoproteins that are involved in post attachment/entry events of virus infection often display cell surface binding activity. The reported neutralisation activity of anti-gp150 antisera, the virion surface location and similarity with EBV gp340/220 of gp150 suggest that gp150 may be involved in MHV-68 attachment and/or entry of target cells. To test this possibility a series of binding experiments was performed using gp150-His.

3.2.7 Application of Gp150-His in a 'Far-western blot'/VOPBA

To identify a cellular protein to which virus and gp150 may bind gp150-His, GST-His, MHV-68 infected cell supernatant and concentrated virus preparations were applied to western blots of different mammalian cell lysates. These included the fibroblastoid BHK cell line and the C127 mouse epithelial cell line which are known to support productive MHV-68 replication. Also included were the transformed cell lines of B cell origin NS0, S11, S31, and A20, since MHV-68 is B lymphotropic. A transformed cell line of T cell origin, EL4, was also included. For each lane of a 7.5% SDS-PAGE gel cell lysate equivalent to 1 x 10^5 cells was loaded, proteins were

Figure 3.4.3 Expression & purification of GST-His from bacteria carrying pGEX-2T/His



Clones of bacteria transformed with pGEX-2T/His were induced to express the fusion protein by addition of isopropyl-ß-D-thiogalactopyranoside (IPTG). **A.** Western blot the whole bacterial lysate of clone 1 prior to induction (lane 1), 30, 90 and 150 minutes post induction (lanes 2, 3 and 4 respectively) probed with an anti-hexahistidine mouse monoclonal antibody followed by a goat anti-mouse alkaline phophatase conjugated secondary antibody. Transferred from a 14% acrylamide SDS-PAGE gel **B.** Samples were taken from each stage of affinity purification of GST-His from bacteria using Ni^{2+} charged resin beads and analysed in a 14% acrylamide SDS-PAGE gel, lane: 1 - uninduced, 2 - induced, 3 - pellet/insoluble fraction, 4 - supernatant/soluble fraction, 5 - supernatant in binding buffer, 6 - binding buffer wash, 7 - wash buffer, 8 - eluate (purified GST-His), 9 - strip buffer, 10 - Ni^{2+} resin beads. The SDS-PAGE gel was stained with Coomassie blue to visualise proteins. Numbers at the left hand side indicate the molecular weight in kDa.

electrophoretically transferred to membranes, strips of which were used for binding analysis. Incubations with the fusion proteins were performed at 4°C and room temperature for times ranging from 1 hour to overnight. To detect binding, anti-gp150 antibody was used as the primary antibody followed by the secondary swine anti-rabbit alkaline phosphatase conjugated antibody. Different cell lines gave rise to different patterns of background staining but no specific bands for either virus or gp150-His were detected. Various concentrations of the gp150 fusion protein (from 51.3ng/ml (11pM) to 51.3μg/ml (11nM)) and of virus from infected cell supernatant (5×10^6 pfu/ml) and also concentrated virus preparations (up to 3×10^8 pfu/ml) were tested. In addition various concentrations (from 1/100 to 1/1000) of the primary antibody were used however specific binding was not demonstrated.

A new strategy was adopted to eliminate the use of antibody in an attempt to reduce background non-specific bands. This was considered a valid option as if there was any specific binding it may have been masked by the non-specific staining. Biotinylated fusion proteins were applied to western blots of cells and an avidin-alkaline phosphatase conjugate was used for detection. The resultant bands were determined to be non-specific. Efforts to eliminate the non-specific binding of the avidin alkaline phophatase conjugate included varying the salt concentration of the Tris-HCl buffer from 150-500mM NaCl, and changing the blocking buffer from milk protein to bovine serum albumin. However, the non-specific binding was not eliminated by these methods and no specific binding of MHV-68 or gp150-His was detected.

The use of a VOPBA (virus overlay protein binding assay) based method was considered a valid approach in an effort to detect binding of virus and gp150-His to cellular proteins. Variations of this technique have proved effective for the identification of putative cell receptors for a number of viruses including reovirus type 3 (Co *et al.*, 1985), mouse hepatitis virus (Boyle *et al.*, 1987), CMV (Adlish *et al.*, 1990, Taylor & Cooper, 1990), lymphocytic choriomeningitis virus (LCMV) (Borrow & Oldstone, 1992), Theiler's virus (Kilpatrick & Lipton, 1991), visna virus (Dalziel *et al.*, 1991, Crane *et al.*, 1991) and encephalomyocarditis virus (Jin *et al.*, 1994). However VOPBAs are only useful in certain circumstances where binding is

not dependent on protein conformation or affected by denaturation, a negative result by VOPBA alone by no means rules out lack of binding function of a virus or protein (Boyle *et al.*, 1987).

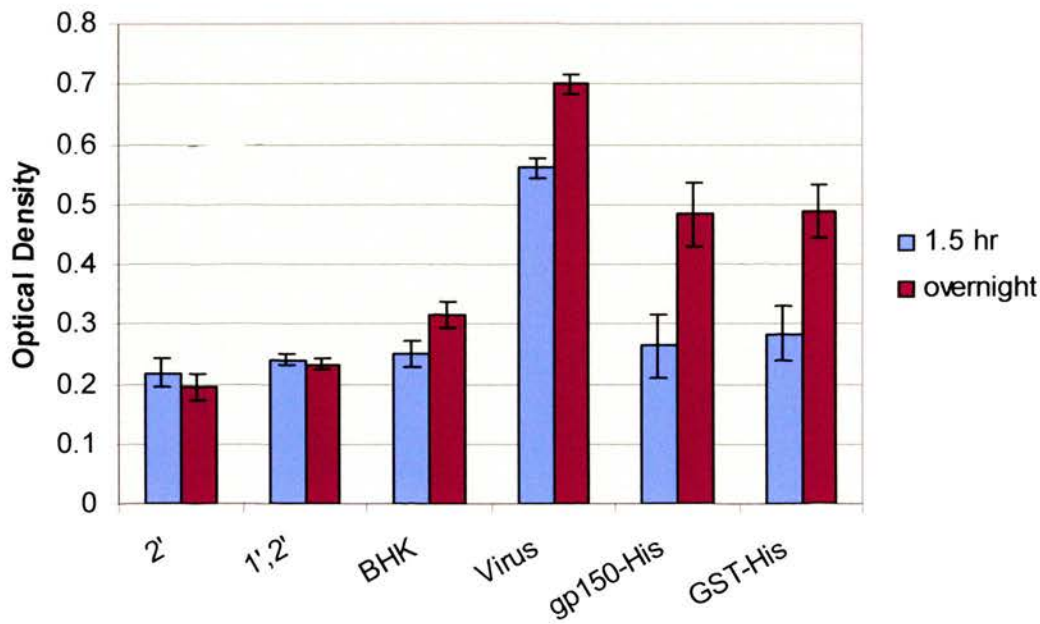
3.2.8 Analysis of Gp150-His Binding to Epithelial Cells - ELISA Binding Assay

To test the ability of gp150-His to bind to C127 murine epithelial cells an enzyme linked immunosorbent (ELISA) based assay was developed. C127 cells were selected as antibodies against gp150 were reported to neutralise MHV-68 infection of these rodent cells (Stewart *et al.*, 1996). The use of this ELISA based system avoided harvesting of cells eliminating mechanical disruption of cells and the use of the protease trypsin which may destroy potential cell surface binding epitopes. The receptors of some viruses such as rhinovirus (Mischak *et al.*, 1988), B-lymphotropic papovavirus (Haun *et al.*, 1993) and papillomavirus (Qi *et al.*, 1996) are destroyed by trypsin.

Confluent monolayers of paraformaldehyde fixed C127 cells (approximately 5 x 10^4 cells/well) in 96 well plates were incubated with equimolar concentrations of each fusion protein, 50µl of 20µM protein solution ($6x10^{14}$ molecules/well). In addition 10^7 pfu/well of MHV-68 as a positive binding control and BHK cell lysate as a virus negative binding control were applied. Incubations of protein and virus with the cells were performed for 1.5 hours or overnight at 37°C and 4°C, respectively. The primary antibody used was rabbit anti-gp150 antiserum followed by swine anti-rabbit alkaline conjugated antiserum. Alternatively, a mouse anti-hexahistidine monoclonal antibody was used followed by a goat anti-mouse alkaline phophatase antibody. Binding assays were repeated three times in triplicates.

As shown in figure 3.4.4 MHV-68 bound to fixed C127 cell monolayers in the ELISA assay. Greater binding was observed the longer the binding incubation time and was greater at 37°C compared to 4°C. The readout for gp150-His binding did not differ significantly from the GST-His control fusion protein. Equivalent results were observed for fusion protein binding levels whether mouse anti-hexahistidine monoclonal antibody or anti-gp150-GST antibody was used for detection. In relation to the control protein any gp150-His binding observed was deemed non-specific.

Figure 3.4.4. Analysis of the ability of gp150-His to bind to C127 mouse epithelial cell monolayers



The level of binding detected for gp150-His applied to paraformaldehye fixed monolayers of C127 epithelial cells in an enzyme linked immunosorbent based assay. Equimolar quantities of fusion proteins (50µl/well of 20µM protein solution), 10^7 pfu of MHV-68/well (virus) or BHK cell lysate (BHK) as a virus negative control, were applied to monolayers in a 96 well plate for either 1.5 hours at room temperature or incubated overnight at 4°C. To detect binding the primary antibody used was rabbit anti-gp150 antibody (1') followed by swine anti-rabbit alkaline phosphatase conjugate (2'). The optical density of wells was measured at 405nm following addition of the alkaline phosphatase substrate. The experiment was performed in quadruplicates, bars denote standard deviation. Chart represents one of three sets of experiments.

The use of the ELISA based assay for analysis of the binding potential of gp150-His to lymphocytes was attempted. However, technical difficulties were experienced due to the characteristic lack of adherence of lymphocytes which ultimately resulted in loss of cells from the wells. In this case cells used included the A20 and NS0 B cell lines and primary splenic lymphocytes. Measures used to avoid the cell loss problem included use of extreme care when washing wells by using slow hand pipetting, precoating wells with anti-mouse immunoglobulin antibody (for purified splenic B cells) and precoating wells with poly-L-lysine. These measures failed to resolve the problem. The failure of the precoating techniques to prove effective was surprising however, even new preparations of antibody applied by standard coating protocols did not enable sufficient cell adherence for the assay. These measures could also have adverse effects on the binding of virus and proteins to the cells as reorganisation of molecules in the cell membrane may occur and the interaction of B cell immunoglobulins with the antibody coating the wells may cause cell activation and therefore upregulation of activation markers on the surface of the cells.

3.2.9 The Ability of Gp150-His to Block Virus infection

The ability of gp150-His to block MHV-68 infection of epithelial cells was tested by plaque assay. C127 monolayers were incubated with fusion protein (2μM) for one hour at 4°C, 200 pfu of virus was applied to the monolayers and incubation was continued for one hour at 4°C and then shifted to 37°C for one hour followed by removal of the virus and washing of the monolayer with a low pH buffer to inactivate and remove remaining extracellular virus. Repeat experiments consistently showed no significant blocking of MHV-68 infection of mouse epithelial monolayers by gp150-His (refer to table 3.1.1). Incubation with gp150-His fusion protein and virus infection using the same quantities was also done with cells (10^6 cells per tube) in suspension however, no blocking of MHV-68 infection was detected.

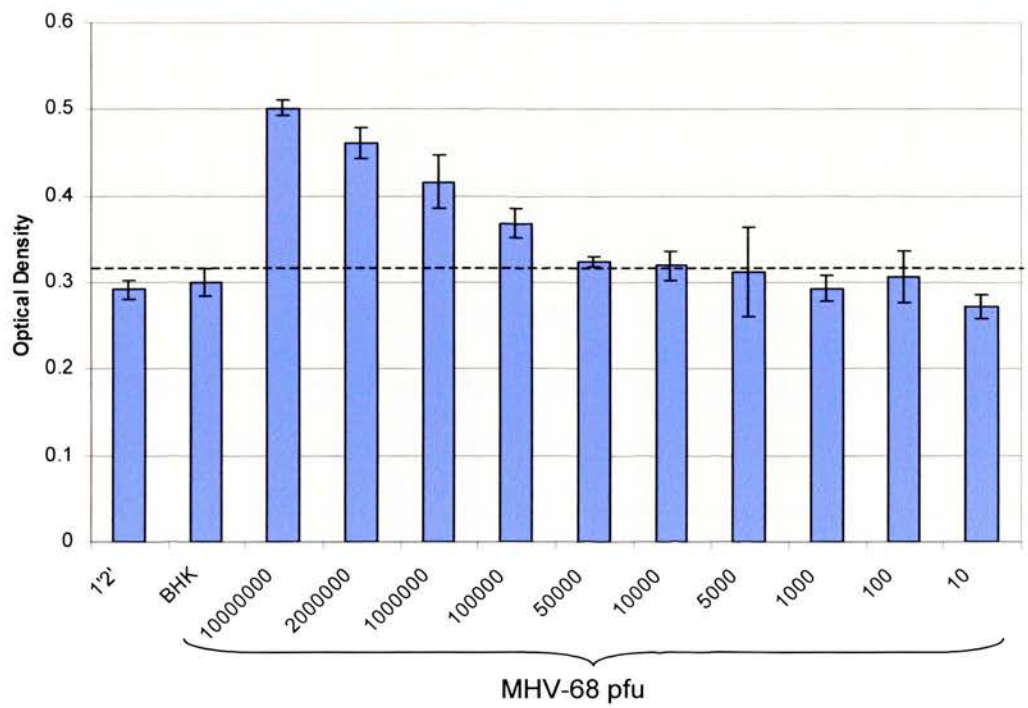
Well	Number of Plaques	
	+ gp150-His	- gp150-His
1	180	280
2	330	210
3	231	212
4	148	188
Mean	222.25 (±79.5)	222.5 (±39.8)

Table 3.1.1. Ability of gp150-His to block MHV-68 infection of C127 epithelial cells. Prior to MHV-68 infection (200pfu), C127 cell monolayers in six well plates were incubated, for one hour, with 500µl media in the presence (+ gp150-His) or absence (- gp150-His) of 2µM gp150-His to determine if gp150-His could block MHV-68 infection. After 5 days post infection monolayers were fixed with formal saline and the number of plaques counted. A representative set of data is shown, the figures in brackets show the standard deviation.

3.3.1 Ability of Antiserum to Block MHV-68 Binding to Epithelial Cells

To investigate if the anti-gp150 antibody generated against a GST-gp150 fusion protein, previously reported to neutralise infection, blocked binding of virus to epithelial cells the ELISA based assay was used. This was in order to elucidate the mechanism of virus neutralisation i.e. to determine if the antibody blocked attachment of virus to cells. Initially, the minimum virus concentration that could be applied in the ELISA assay and detected as positive binding virus was tested. This was done in order to be able to optimise the ratio of antibody to virus that may allow even weak antibody block of virus binding to be detected. Virus was applied in a series of dilutions to monolayers of C127 cells in 96 well plates. Anti-gp150 antibody was used to detect binding of virus, followed by the swine anti-rabbit alkaline phophatase conjugated secondary. As figures 3.4.5 shows the lowest virus concentration applied to C127 cells that could be detected above background was 1 x 10^5 pfu . It was decided that 10^7 pfu per well would be used, as this shows clear binding, the lower concentrations that allowed detection of positive virus binding would perhaps be too low to reasonably avoid the possibility of ambiguous results.

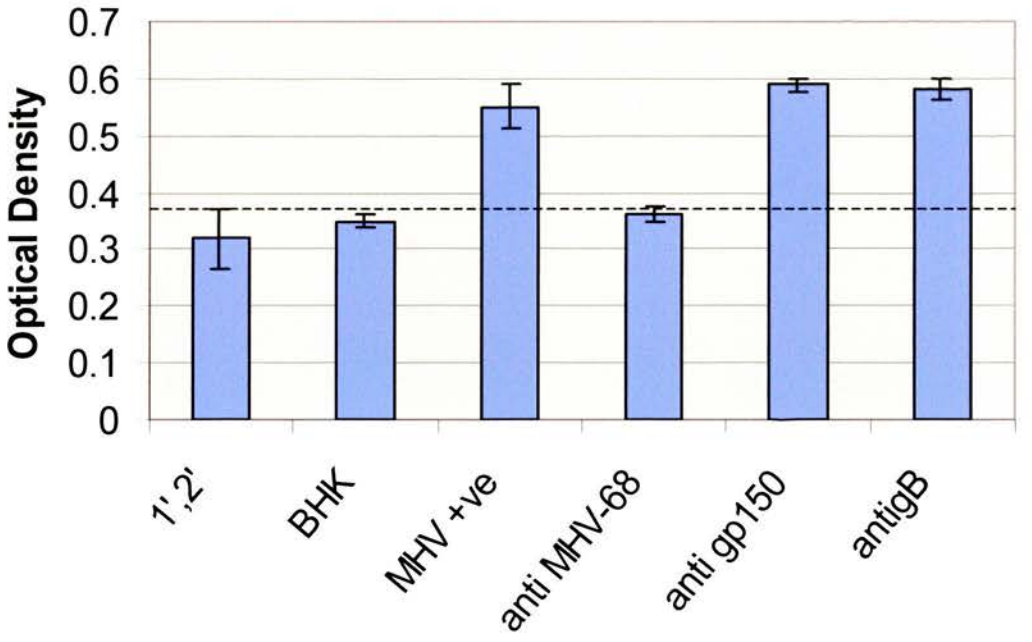
Figure 3.4.5. Analysis of the minimum level of MHV-68 virus that results in detection of positive binding above background



MHV-68 was diluted in series and applied to paraformaldehyde fixed C127 mouse epithelial monolayers in order to determine the minimum virus required to detect positive binding above background in an enzyme linked immunosorbent based assay. BHK cell lysate (BHK) was used as a virus negative control. The primary antibody (1') used for the detection of binding was rabbit anti-gp150 antibody followed by a swine anti-rabbit alkaline phosphatase conjugate (2'). Following the addition of the alkaline phosphatase substrate the optical density was measured at 405nm. Quadruplicates were done, the bars denote standard deviation. The dotted line indicates the cut off point for detection of significant binding above background.

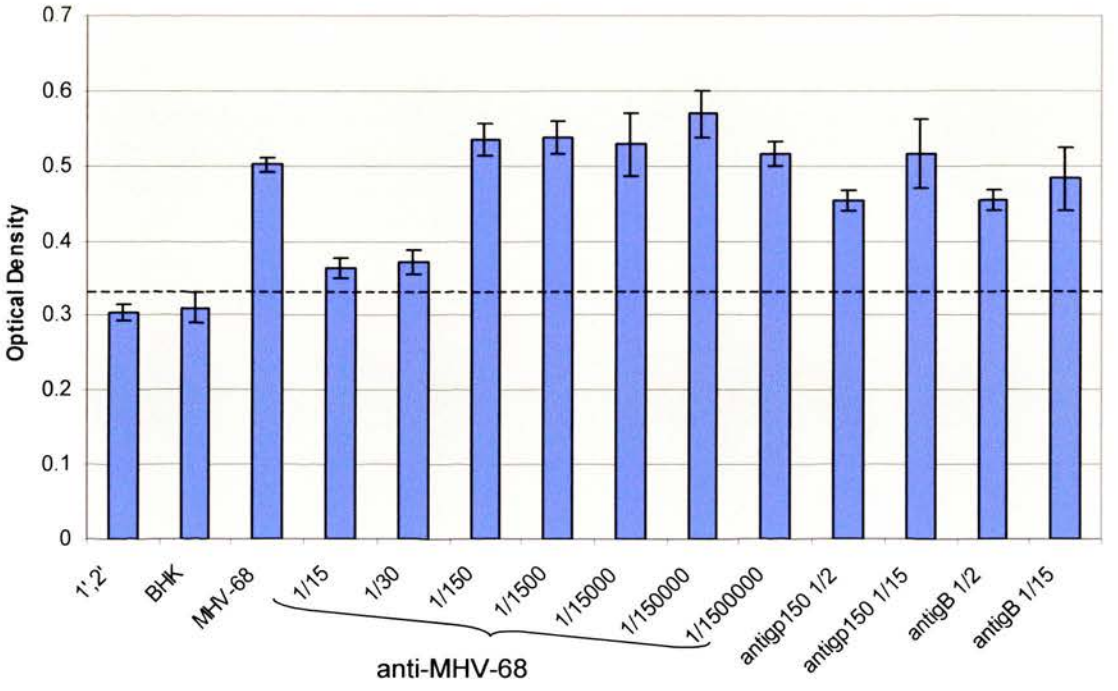
Next, virus (10^7pfu) was incubated with dilutions of anti-gp150 antibody, anti-MHV-68 antibody, as a positive antibody blocking control or anti-gB antibody as a negative control. Following an hour incubation with antisera the virus was applied to C127 monolayers in 96 well plates for one hour. As figures 3.4.6a & 3.4.6b show anti-gp150 antibody did not appear to specifically block binding of virus to cells even at very low dilutions of 1/2 and 1/10. The anti-MHV-68 antibody (at 1/10) consistently blocked MHV-68 binding, a feature which could be diluted out as figure 3.4.6b shows. At a dilution of 1/150 the MHV-68 antibody appeared to no longer reduce virus binding. As a negative control the anti-gB antibody did not block binding of MHV-68. This was repeated three times with consistent results.

Figure 3.4.6a. Analysis of the ability of anti-gp150 antisera to block binding of MHV-68 to C127 mouse epithelial cell monolayers



The relative amount of MHV-68 binding to monolayers of paraformaldehye fixed C127 epithelial cells in a 96 well plate following virus incubation with antisera (final dilution 1/10) of different specificities for 1 hour. Virus preincubated with rabbit antisera of different reactivities (anti-MHV-68, anti-gp150 and anti-gB) was applied at 10^7 pfu per well and incubated for 1.5 hours at room temperature. For detection of binding the primary antibody used was rabbit anti-gp150 antibody (1') followed by a swine anti-rabbit alkaline phosphatase conjugated antibody (2'). The optical density of wells was measured at 405nm following addition of the alkaline phosphatase substrate. BHK cell lysate (BHK) was used as a virus negative control, BHK cell lysate preincubated with the different antisera was also done however no difference from BHK lysate alone was observed. MHV-68 +ve = virus alone (not incubated with antibody), as a positive binding control. Bars denote standard deviation of quadruplicate samples. Chart represents one of two experiments. The dotted line indicates the cut off point for positive binding above background.

Figure 3.4.6b. Analysis of the level of anti-MHV-68 antibody necessary to block MHV-68 binding to C127 mouse epihelial cell monolayers



Determination of the level of antibody required to block binding of MHV-68 to C127 mouse epithelial cells. MHV-68 virus (10^7pfu/well) was incubated with the sera dilutions indicated for 1 hour prior to application of virus to monolayers of paraformaldehye fixed C127 cells in 96 well plates. An enzyme linked immunosorbent based detection system was used, the primary antibody (1') was rabbit anti-gp150 antiserum followed by a swine anti-rabbit alkaline phosphatase conjugated secondary antibody (2'). The alkaline phophatase substrate BCIP/NBT was used and the optical density read at 405nm. Bars denote standard deviation of quadruplicate wells. Chart represents one of two experiments. Note: higher dilutions of anti-gp150 and anti-gB (i.e. 1/100, 1/500 & 1/1000, 10,000) were done however similar results to lower dilutions were obtained therefore bars not shown. BHK cell lysate (BHK) was used as a virus negative control. The dotted line denotes the cut off point for positive binding detection above background.

3.3.2 Virus Neutralisation By Antisera

Following the discovery that the anti-gp150 serum did not block MHV-68 binding to C127 epithelial cells, neutralisation assays, in the form of a conventional plaque reduction assay, were done. Prior to application to C127 epithelial cell monolayers 100pfu of virus was incubated with dilutions (1/2, 1/10 and 1/100) of anti-gp150 antisera. Polyclonal anti-MHV-68 was used as a positive control and anti-gB was used as a negative control for neutralisation. In contrast to previously reported findings (Stewart *et al.*, 1996) the anti-gp150 antibody did not exhibit significant neutralising ability even at very low antibody dilutions of ½ and 1/10. The anti-MHV-68 antibody consistently neutralised MHV-68 plaque formation 100% at a dilution of 1/100 and the anti-gB antibody did not reduce plaque numbers as predicted. Table 3.1.2. shows a representative set of data for antibody neutralisation assays.

MHV-68 alone	**MHV-68 + anti-MHV-68**	**MHV-68 + anti-gp150**	**MHV-68 + anti-gB**
Number of Plaques			
112	0	117	115
105	0	130	118
98	0	120	92
120	0	107	120
109 (±9.43)	**0**	**118.5** (±9.47)	**111** (±13.0)

Table 3.1.2. Analysis of the virus neutralising ability of anti-gp150 antibody by plaque assay. MHV-68 (100pfu) was incubated with rabbit antisera (1/10 final dilution) with different specificities for one hour prior to application to monolayers of C127 epithelial cells in 6 well plates. The sera was heat treated prior to use in order to inactivate complement. After five days post infection cell monolayers were fixed with formal saline and the number of plaques counted. The bottom row indicates the mean number of plaques of quadruplicate wells with the standard deviation in brackets.

3.3.3 Ability of Gp150-His to Bind to Splenic Lymphocytes

In view of the B cell tropism and splenic site of latency displayed by MHV-68 investigations into the ability of gp150-His to bind to splenic lymphocytes was pursued by using fluorescence associated cell sorting (FACS) analysis. Equimolar concentrations of fusion proteins (20μM), 10^7 pfu of MHV-68 as a positive binding

control and BHK cell lysate as a negative virus control were each incubated with 10^6 freshly prepared live BALB/c splenocytes per well for 1.5 hours at 37°C in the presence of sodium azide to inhibit capping. Binding was detected using anti-gp150 antibody followed by a goat anti-rabbit FITC conjugated antibody or anti-hexahistidine monoclonal antibody followed by a sheep anti-mouse FITC conjugated antibody. Binding to splenic lymphocytes was analysed by FACS these cells were discriminated by cell size and granularity. In conjunction to each cell binding assay a proportion of the splenocytes used were analysed by FACS to determine the lymphocyte cell make up of the population. Figure 3.4.9.a shows a representative set of a splenocyte population profile.

The establishment of this assay was delayed by the great loss of cells experienced during the washes between incubations. This technical difficulty was particularly apparent due to the number of washes required in total i.e. 11 washes. The use of standard FACS tubes and vacuum pump suction for removal of supernatant between centrifugal pelleting of cells was abandoned. Ultimately, round bottom 96 well plates were used, centrifugation was done for five minutes at 500 *x g* with the brake off and the supernatant removed by careful hand pipetting to avoid dislodging cells.

As figure 3.4.7 shows significant binding of lymphocytes by gp150-His at 20μM in comparison to GST-His was detected, in contrast to the epithelial cell binding data. A shift to the right was repeatedly seen on FACS graphs indicating cells with increased FITC staining when gp150-His was applied to splenocytes. Similar results were obtained using either pair of antibodies for detection. MHV-68 was consistently determined to bind approximately 10% of the lymphocytes. Gp150-His (at 20μM) repeatedly bound approximately 2 – 3% of lymphocytes a level which was determined >95% significant in comparison to the control GST-His fusion protein by Student's t test. This result could be diluted out, at 200nM of gp150-His protein no significant binding was detected and showed that the levels of protein used were near the threshold between detection of binding and no detection. Preferably, higher concentrations of proteins would have been tested however the quantity of protein used was limited to a maximum of 20μM due to the restricted

amount available. Protein concentrations used were at the upper limit of that which was feasible from a bulk stock of protein that was generated with the intention of maintaining the protein applied across assays consistently from the same stock.

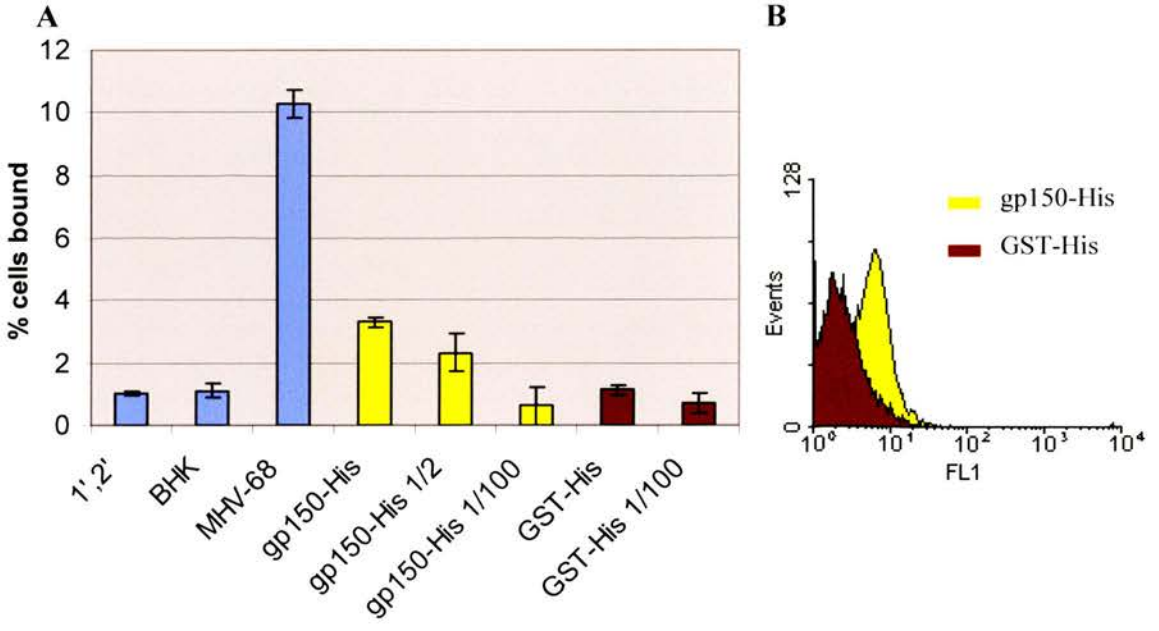
The shape of the FACS graph in figure 3.4.7 for splenic lymhphocyte binding suggests general binding of gp150-His to the heterogeneous population of lymphocytes rather than a specific subset of cells which might show as a second peak rather than a shift in fluorescence of the whole population.

Binding of MHV-68 to primary splenic lymphocytes was also detected by FACS analysis using the polyclonal rabbit anti-MHV-68 antibody as the primary antibody which gave results consistent with those realised using the anti-gp150 antibody as primary antibody i.e. when 10^7 pfu of virus was applied to 10^6 splenocytes approximately 10% of the lymphocytes were bound by virus (see figure 3.4.8).

3.3.4 Ability of Gp150-His to Bind to Purified Splenic B cells

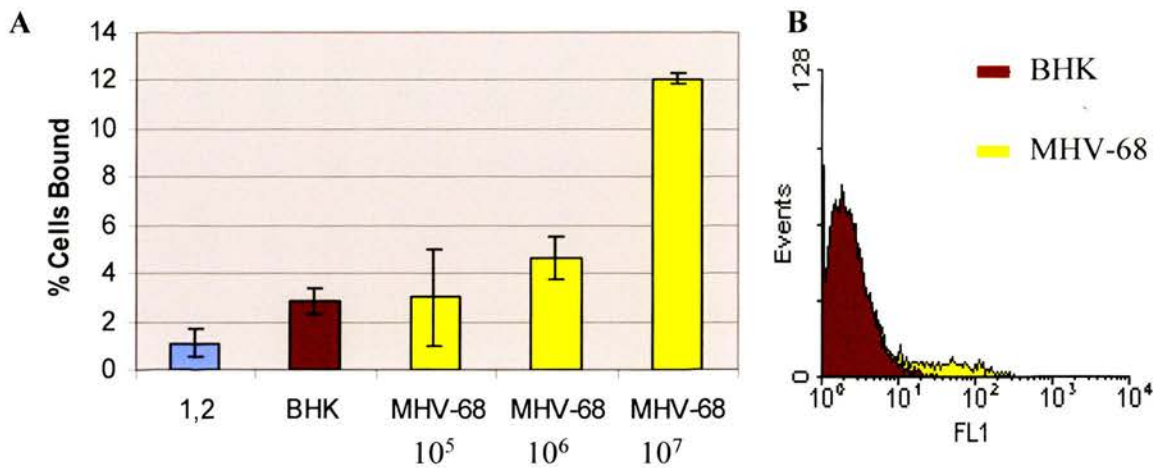
In view of results indicating gp150-His binding to splenic lymphocytes the fusion proteins were applied to purified B cells. Of the subsets, B cells were selected for analysis of potential gp150-His binding on the basis that MHV-68 establishes latent infection in B cells. In addition gp150 may be functionally similar to gp340 of EBV that specifically binds B cells. B cells were purified from freshly obtained splenocytes by magnetic associated cell sorting (MACS) depletion, an anti-CD43 monoclonal antibody conjugated to metal beads was employed to specifically retain cells expressing the CD43 epitope. The CD43 antigen is expressed on IL-7 responsive pro-B cells, plasma cells, peritoneal and splenic $CD5^+$ B cells (B-1 cells), granulocytes, monocytes, macrophages, platelets, NK cells, thymocytes, peripheral cytotoxic T cells and most helper T cells. CD43 is not expressed on resting conventional peripheral B cells (Yedidia *et al.*, 1998). This method routinely gave preparations that were >90% pure as determined by FACS staining for lymphocyte subsets (see figure 3.4.9). The FACS based splenic B cell binding assay was performed as for the whole splenocyte binding assay. As figure 3.5.1 shows significant binding of gp150-His to approximately 8% of splenic B cells was

Figure 3.4.7. Flow cytometric analysis of the ability of gp150-His to bind to BALB/c splenic lymphocytes



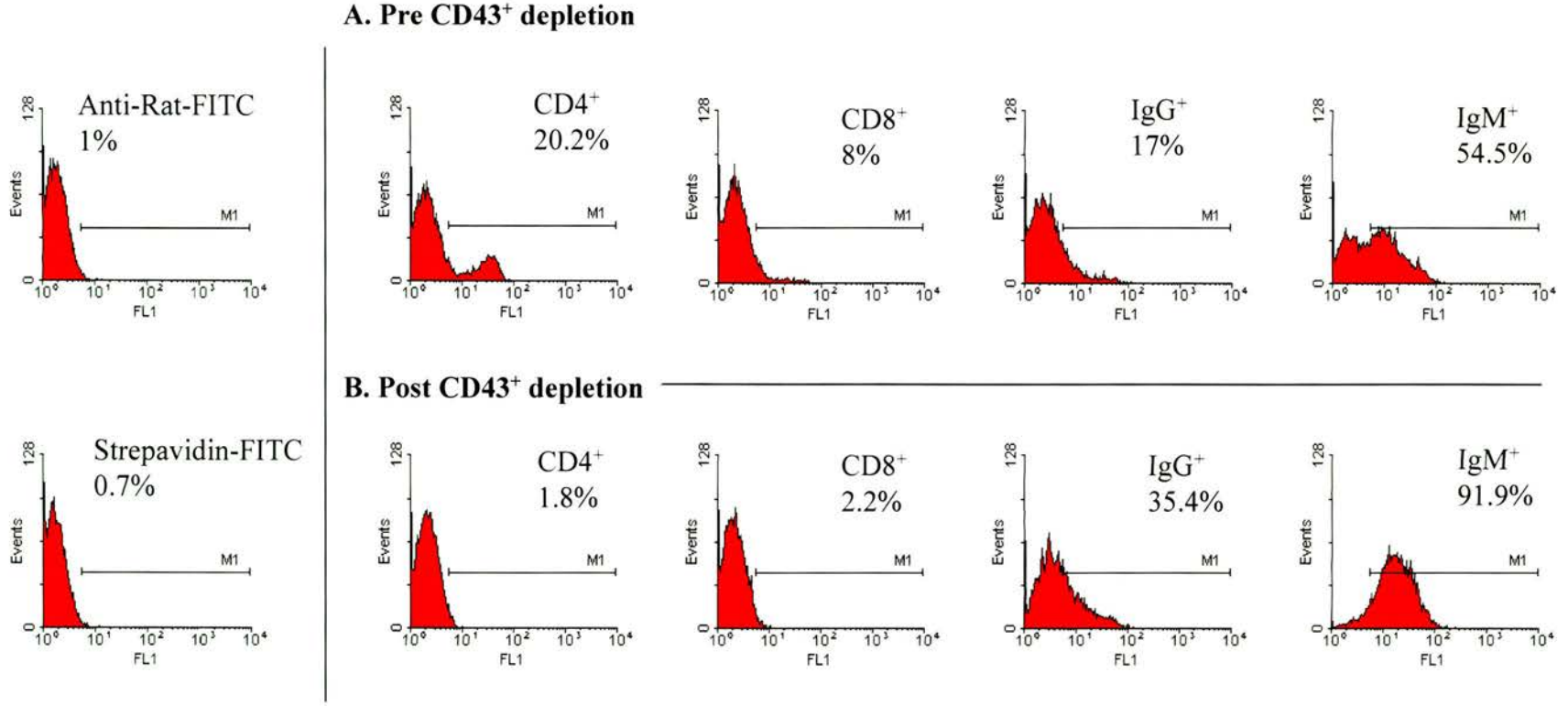
A. Freshly prepared live BALB/c splenocytes (10^6/well) were incubated, in suspension, with equimolar concentrations (20 μM) of each fusion protein (gp150-His or GST-His), 10^7 pfu of MHV-68 or BHK cell lysate (BHK) as a negative virus control for 1.5 hours at 37°C in the presence of 0.2% sodium azide. The primary antibody used for detection of binding was rabbit anti-gp150 antibody (1') followed by swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody (2'). Cells were formaldehyde fixed and analysed by fluorescence associated cell sorting (FACS). Lymphocytes were discriminated by cell size and granularity. The marker for positive staining was positioned at 1% of staining with 1',2' antibody alone. The bars denote standard deviation of triplicate samples. Chart represents one of three sets of experiments. **B.** Representative GST-His scatter profile of lymphocytes superimposed on top of a gp150-His profile.

Figure 3.4.8. Flow cytometric analysis of MHV-68 binding to BALB/c splenic lymphocytes



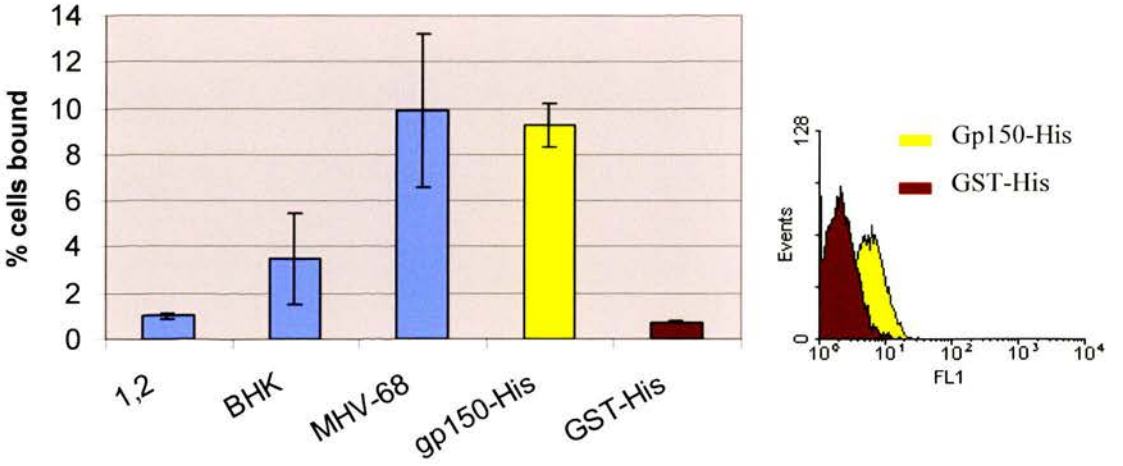
A. Dilutions of MHV-68 were incubated with 10^6 primary murine splenocytes per well for 1.5 hours at 37°C in the presence of sodium azide. A baby hamster kidney cell lysate preparation (BHK) was used as a virus negative control at a concentration equivalent to the highest concentration of MHV-68 applied, figures below MHV-68 on x axis indicate the total pfu of virus applied per well. The primary antibody used was polyclonal rabbit anti-MHV-68 antibody (1) followed by swine anti-rabbit flouresceine isothiocyanate (FITC) conjugated antibody (2). Bars denote standard deviation from triplicate samples. MHV-68 binding observed when a total pfu of 10^7 was applied in comparison to the BHK control is 99.8% significant as determined by Student's t test. Chart represents data from one of two experiments. **B.** Representative BHK FACS scatter profile of splenocytes superimposed on top of a MHV-68 (10^7 pfu applied) profile.

Figure 3.4.9. Flow cytometric analysis of lymphocyte populations of BALB/c splenocytes pre and post depletion of CD43$^+$ cells



Representative set of graphs generated by fluorescence associated cell sorting (FACS) analysis of BALB/c splenocytes prior to (A) and post (B) magnetic associated cell sorting (MACS) depletion of CD43$^+$ cells i.e. enrichment of mature B cells. Cells were immunostained with directly or indirectly fluoresceine isothiocyanate (FITC) labeled antibodies against the indicated lymphocyte cell surface markers. For FACS, lymphocytes were discriminated for by cell size and granularity. The marker (M1) for positively stained cells was positioned at approximately 1% of background staining, percentage of cells within the marker is indicated. Antibodies used included rat anti-CD4 and rat anti-CD8 detected by anti-rat FITC conjugate, biotinylated anti-IgM followed by a strepavidin FITC conjugate and FITC conjugated anti-IgG.

Figure 3.5.1. FACS analysis of the ability of gp150-His to bind to purified splenic B cells



A. Primary B cells were purified from BALB/c splenocytes by magnetic cell sorting (MACS) depletion of $CD43^+$ cells. Purified B cells (10^6/well) were incubated with an equimolar concentration (20μM) of each fusion protein (GST-His or gp150-His), 10^7 pfu MHV-68 and BHK cell lysate (BHK) as a negative virus control for 1.5 hours at 37°C in the presence of sodium azide. The primary antibody used for detection of binding was rabbit anti-gp150 antibody(1) followed by a swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated secondary (2). Bars show standard deviation of triplicates. Chart shows representative data from one of two experiments. **B.** Representative FACS profiles of B cells incubated with each fusion protein, GST-His profile superimposed on that of gp150-His.

observed. Approximately 6% of purified B cells were bound by MHV-68 in comparison to control BHK cell lysate staining.

3.3.5 CD19 Phenotype of Cells Bound by Gp150-His & MHV-68

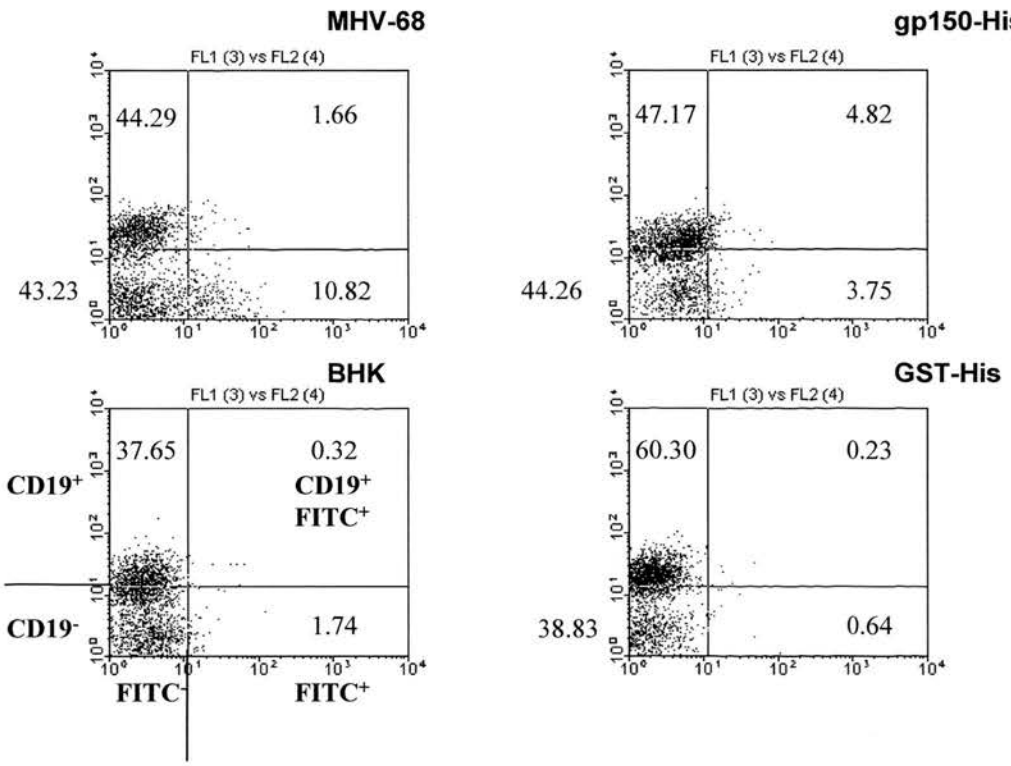
In order to further analyse the lymphocyte cell types bound by MHV-68 and gp150-His double FACS staining was done. Fusion proteins and virus were applied to BALB/c splenocytes and then antibodies for detection of the fusion protein were applied in conjunction with a primary rat antibody for CD19 and a secondary goat anti-rat phytoerythrin conjugated antibody. CD19 is a B cell specific marker expressed throughout B cell development from the pro-B cell through to the mature B cell stages (Krop *et al.*, 1996, Krop *et al.*, 1996b, Fearon, 1993, Tedder *et al.*, 1994). Terminally differentiated plasma cells do not express CD19. On the surface of mature B cells the CD19 molecule associates with CD21(CR2) and CD81 (TAPA-1) and this multimolecular complex synergizes with surface immunoglobulin to promote cellular activation (Krop *et al.*, 1996b, Fearon, 1993, Tedder *et al.*, 1994).

The FACS data indicate that both $CD19^+$ and $CD19^-$ subsets of cells bind virus and gp150-His (see figures 3.5.2a). Total cell binding of gp150-His and MHV-68 in comparison to the appropriate controls are significant by 95% and 99.9% respectively by Student's t test. Of the splenocytes that gp150-His binds approximately 52% are $CD19^+$ and 48% are $CD19^-$. Of the splenocytes that stained positive with incubation with MHV-68 approximately 15% are $CD19^+$ and 85% are $CD19^-$ (see figure 3.5.2b).

3.3.6 FACS Analysis of Gp150-His ability to Bind C127 Epithelial Cells

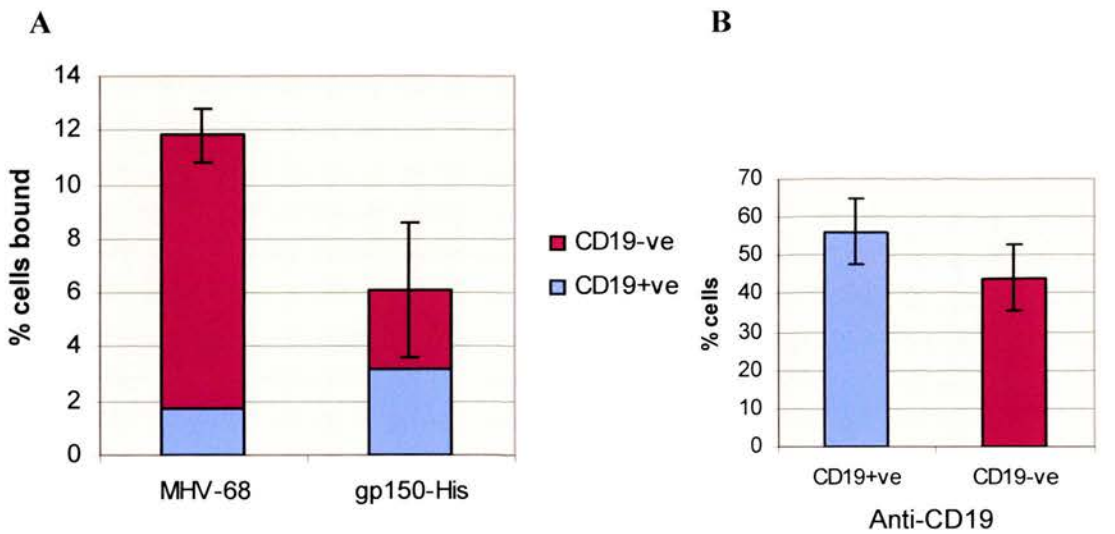
With the establishment of the FACS binding assay C127 epithelial cells were put through the system using the same conditions as for splenocytes. Consistent with the ELISA binding data gp150-His failed to show significant binding from that of the control protein. In contrast, MHV-68 demonstrated binding to approximately 58% of the cells (refer to figure 3.5.3).

Figure 3.5.2a. CD19 phenotype of BALB/c splenic lymphocytes bound by MHV-68 and gp150-His determined by FACS analysis



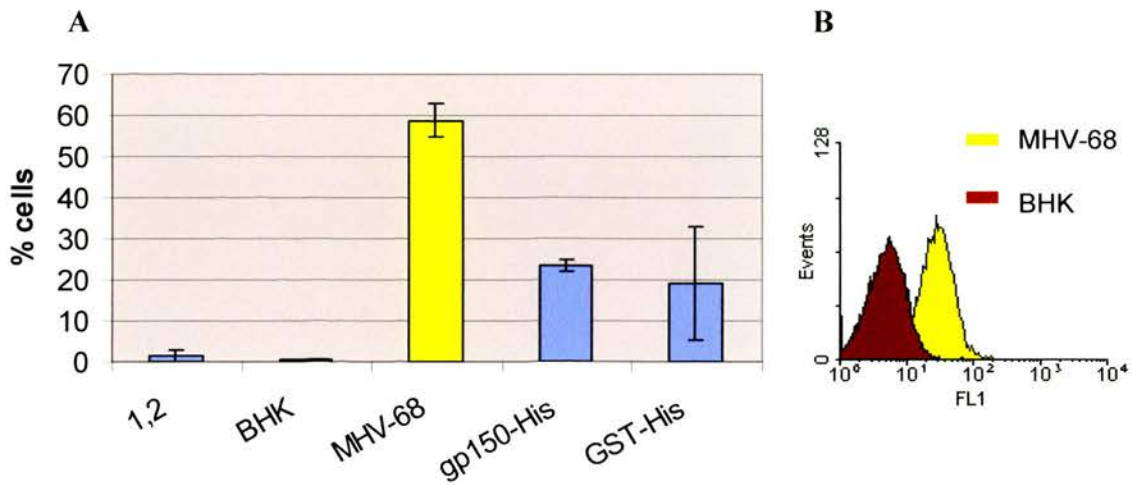
Primary BALB/c splenocytes (10^6/well) were incubated with an equimolar concentration (20μM) of fusion proteins (GST-His or gp150-His), 10^7 pfu of MHV-68 and BHK cell lysate (BHK) as a negative virus control, for 1.5 hours at 37°C in the presence of sodium azide. Double staining was done, primary antibodies used were rabbit anti-gp150 antibody and rat anti-CD19 antibody, secondary antibodies used were swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody and goat anti-rat R-phytoerythrin. The markers for positively stained cells were set at approximately 1% of anti-gp150 antibody plus anti-rabbit FITC on the x axis and at 1% of anti-Rat PE alone on the y axis. Figures show representative FACS scatter profiles for splenic lymphocytes incubated with the indicated reagent, numbers indicate the percentage of cells in each quadrant. Labels in the bottom left panel indicate phenotype staining of cells in each quadrant. Assays were done in triplicate.

Figure 3.5.2b. CD19 phenotype of BALB/c splenic lymphocytes bound by MHV-68 and gp150-His



Primary BALB/c splenocytes (10^6/well) were incubated with an equimolar concentration (20μM) of fusion proteins (GST-His and gp150-His), 10^7 pfu of MHV-68 and BHK cell lysate (BHK) as a negative virus control, for 1.5 hours at 37°C in the presence of sodium azide. Double staining was done, primary antibodies used were rabbit anti-gp150 antibody and rat anti-CD19 antibody, secondary antibodies used were swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody and goat anti-rat R-phytoerythrin (PE) conjugated antibody. Fluorescence associated cell sorting (FACS) analysis was done, lymphocytes were discriminated for by cell size and granularity. The markers for positively stained cells were set at approximately 1% of anti-gp150 antibody plus anti-rabbit FITC and anti-Rat PE alone. **A.** The chart displays the percentage of lymphocytes bound by gp150-His and MHV-68 and the proportion of CD19+ve and CD19-ve cells within the bound population. **B.** The overall CD19 phenotype of the whole population of lymphocytes. The bars denote standard deviation of triplicates. Representative data from one of two experiments.

Figure 3.5.3. FACS analysis of the ability of MHV-68 & gp150-His to bind to C127 murine epithelial cells

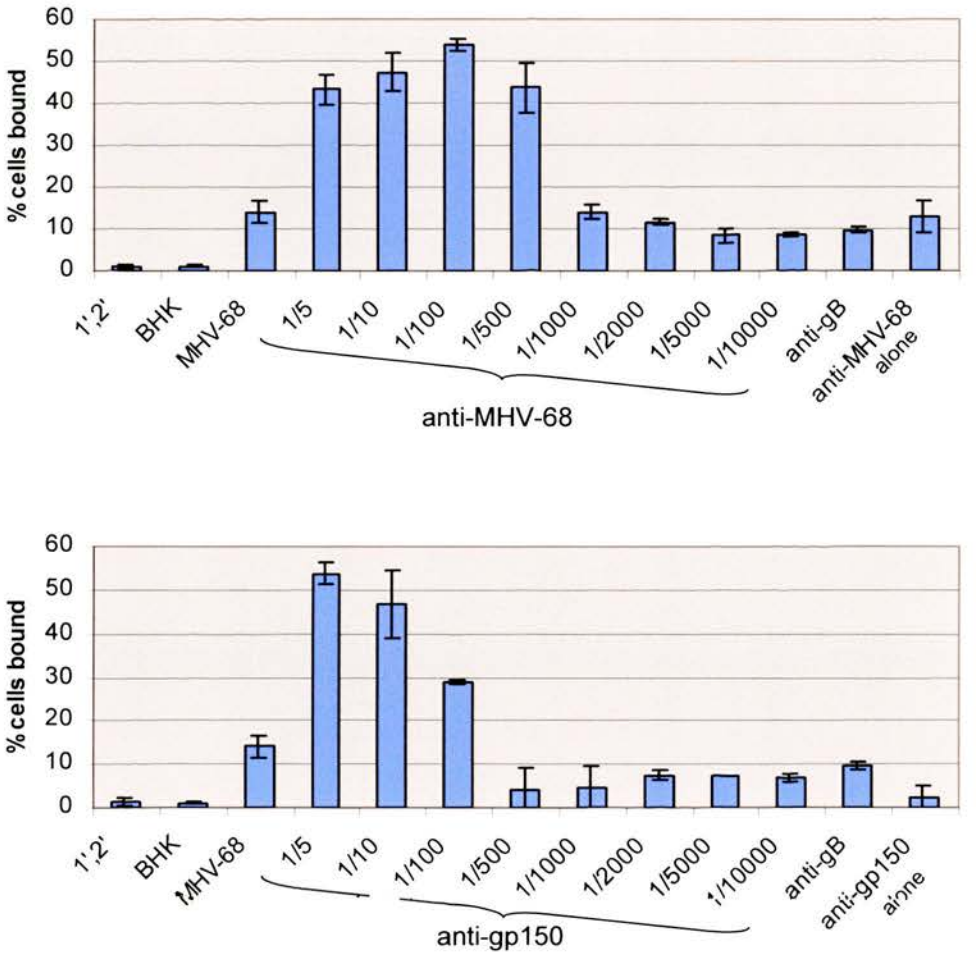


A. C127 murine epithelial cells (10^6/well) were incubated with equimolar concentrations (20μM) of fusion protein (gp150-His or GST-His), 10^7 pfu MHV-68 or BHK cell lysate (BHK) as a virus negative control, for 1.5 hours at 37°C in the presence of sodium azide. The primary antibody used for detection of binding was rabbit anti-gp150 antibody (1) followed by swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody (2). Cells were formaldehyde fixed and analysed by fluorescence associated cell sorting (FACS). The marker for positive staining was positioned at 1% of staining with 1,2 antibody alone. The bars denote standard deviation of triplicate samples. **B**. Representative FACS profile of MHV-68 overlayed with a virus negative control BHK cell lysate profile.

3.3.7 FACS Analysis of the Effect of Preincubation of MHV-68 with Antisera on Virus Binding to BALB/c Splenic Lymphocytes

The effect of preincubation of virus with antisera prior to application to splenic lymphocytes was tested. MHV-68 (10^7 pfu) was incubated with increasing dilutions of antisera with different specificities (anti-gp150, anti-MHV-68 and anti-gB) for one hour prior to incubation with primary BALB/c splenocytes (10^6/well) for 1.5 hours at 37°C in the presence of sodium azide. Antibodies used for detection of virus binding were rabbit anti-gp150 antibody followed by swine anti-rabbit FITC conjugated antibody. FACS analysis was done, lymphocytes were discriminated by cell size and granularity. As figure 3.5.4 shows, at antisera dilutions of 1/5 up to 1/100 and 1/500 increased numbers of cells were bound by virus over that observed for virus alone, were detected with either anti-gp150 and anti-MHV-68 antisera, respectively. With higher dilutions of anti-gp150 and anti-MHV-68 antibody (<1/1000) the level of virus binding observed was similar to the level measured with virus alone that had not been incubated with antibody. This phenomenon was seen in repeat experiments and is thought to be due to antibody coated virus attaching to lymphocytes via Fc receptors. The anti-gB antibody did not significantly alter virus binding in comparison to MHV-68 alone as determined by Student's t test.

Figure 3.5.4. FACS analysis on the effect of preincubation of MHV-68 with antisera on virus binding to BALB/c splenic lymphocytes



MHV-68 (10^7pfu) was incubated with dilutions of rabbit antisera with the specificities indicated for one hour prior to incubation with BALB/c splenocytes (10^6/well) for 1.5 hours at 37°C in the presence of sodium azide. The primary antibody used for detection of binding was rabbit anti-gp150 antibody (1') followed by swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody (2'). Cells were formaldehyde fixed and analysed by fluorescence associated cell sorting (FACS). Lymphocytes were discriminated by cell size and granularity. The marker for positive staining was positioned at 1% of staining with 1',2' antibody alone. The bars denote standard deviation of duplicate samples. BHK- BHK cell lysate as a virus negative control, anti-gB- anti-gB antibody at a dilution of 1/10, MHV-68- MHV-68 alone, not incubated with antibody. The far right column for both charts shows the indicated antisera (1/10) incubated with cells in the absence of virus.

3.3.8 GP150 BASED VACCINE

MHV-68 provides the opportunity to investigate the potential of a glycoprotein based vaccine for gammaherpesviruses in an amenable animal model. As described in sections 1.8.7 – 1.8.9 much emphasis has been put on the possible use of gp340/220 of EBV as a vaccine immunogen. A wide array of vaccine forms have been pursued and there has been limited success in animal models and small human trials with a recombinant vaccinia virus expressing gp340 (Morgan *et al.*, 1988b, Mackett *et al.*, 1996, Gu *et al.*, 1995). Gp150 has been proposed as a candidate vaccine antigen for immunisation against MHV-68 infection of mice. MHV-68 vaccination studies are in their infancy however, at least three vaccination strategies have been reported. A recombinant vaccinia virus expressing gp150 (VV^{gp150}) administered subcutaneously to mice resulted in the production of gp150 neutralising antibodies and upon subsequent MHV-68 challenge splenomegaly and the peak level of latent virus in the spleen was greatly reduced (Stewart *et al.*, 1999a). In addition, mice immunised with *in vitro* cultured dendritic cells pulsed with MHV-68 lytic cycle $CD8^+$ and $CD4^+$ T cell epitopes (including one derived from gp150) experienced significantly reduced (10-100 fold) lung viral titres following MHV-68 infection (Liu *et al.*, 1999). An alternative strategy to prime $CD8^+$ T cells against MHV-68 has been done using a recombinant vaccinia virus and a recombinant influenza A virus both engineered to express MHV-68 lytic phase CTL epitopes (Stevenson *et al.*, 1999b). Only partial protection against acute infection was achieved in these studies and none of these vaccination approaches was able to prevent the long-term establishment of MHV-68 latency.

In view of the potential of gp150 to elicit neutralising antibodies and prime a T cell mediated response against lytic antigens it was considered worthwhile to pursue a vaccine based on this glycoprotein. The aim was to develop a strategy focusing on limiting the acute infection and in turn prevent establishment of latency by blocking spread of infectious virus. With consideration of the known immunological control of MHV-68 (refer to section 1.3.8), materials available and recent reports describing the efficacy of DNA vaccination followed by vaccinia virus boost at eliciting high levels of specific cell mediated and humoral immunity for HIV, a vaccine strategy

was formulated (Hanke *et al.*, 1998 & 1999). The aim was to immunise first with a plasmid encoding gp150 to prime animals and then boost the immune response with a recombinant vaccinia virus expressing gp150. The ultimate readout was to determine whether this conferred protection against MHV-68 infection in the acute stage and in the establishment of latency. This strategy was attractive due to the potential to elicit cell-mediated as well as humoral immune responses against MHV-68. In addition co-administration of a plasmid encoding type I interferon alpha with a gp150 encoding plasmid was proposed to see if expression of IFN-αI would enhance the protective effect of immunity generated to the viral antigen gp150. There is evidence to suggest that IFN I plays an important role in the generation and survival of antigen specific $CD8^+$ T cells during a primary immune response. Furthermore, IFN I is thought to promote the survival of pre-existing memory $CD8^+$ T cells (Tough *et al.*, 1996a & b).

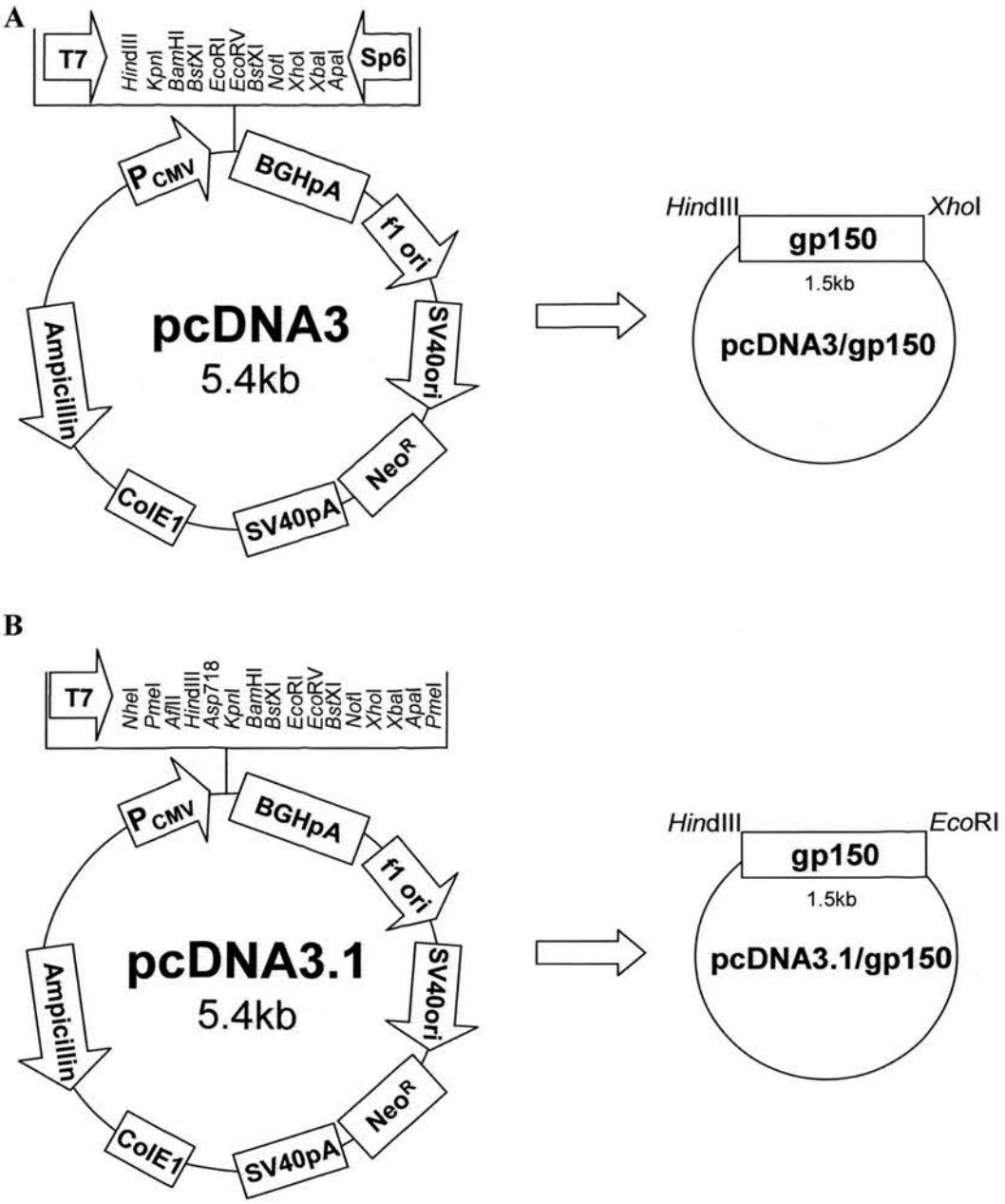
A source of recombinant vaccinia virus expressing gp150 was available. Facilities within the department offered the opportunity for gene gun mediated delivery of the DNA vaccine into the dermis where many dendritic cells known as Langerhans cells reside making the skin a particularly responsive site for vaccination. Following gene gun immunisation dendritic cells resident in the skin are not only capable of taking up, processing and presenting antigen to T cells but they have also been shown to undergo direct transfection with the introduced DNA (Condon *et al.*, 1996, Klinman *et al.*, 1998). Within lymph nodes draining the targetted skin directly transfected cells have been shown to perform a predominant proportion of antigen presentation over un-transfected dendritic cells that have taken up antigen, (Porgador *et al.*, 1998). Another benefit of this method of DNA administration is that 100 – 1000 fold less DNA (levels of approximately 0.4- 2μg) is required in comparison to intramuscular injection (Eisenbraun *et al.*, 1993, Fuller & Haynes, 1994, Barry *et al.*, 1995).

3.3.9 Plasmid Construction for Gp150 Expression

Prior to initiating the proposed vaccination strategy the generation of a plasmid vector encoding gp150 for expression in mammalian cells was required. There are a number of vectors that have been employed for DNA vaccination studies including pcDNA3 (see figure 3.5.5a). Within pcDNA3 the CMV major immediate early promoter/enhancer region drives constitutive expression of inserted genes in eukaryotic cells *in vitro* and *in vivo*. The entire gp150 encoding gene was amplified by PCR using primers IE1gp150 and IE2gp150 (for primers & conditions see section 2.1.8). IE1gp150 includes a Kozak consensus sequence for proper expression of the protein in eukaryotic cells (Cavener & Ray, 1991). The ~1481bp product of these primers was cut with restriction enzymes *Hin*dIII and *Xho*I. Purified cut PCR product was ligated into pcDNA3 that had been prepared by digestion with *Hin*dIII and *Xho*I followed by dephosphorylation to prevent self ligation. The ligation mix was used to transform competent XL1 blue *E.* coli by the heat shock method. All five colonies that grew, selected using ampicillin, were grown up overnight in liquid culture and their plasmid DNA extracted. Digestion of the isolated plasmid DNA with *Hin*dIII and *Xho*I showed one plasmid contained the gp150 insert by the appearance of two bands – one of ~5.4kbp and another of ~1.5kbp. This plasmid was named pcDNA3/gp150. This clone was grown up in bulk liquid culture and a large DNA preparation was made using the CsCl gradient method.

Following confirmation of the identity of the large DNA plasmid preparation by the same digestions used previously the plasmid was transfected into mammalian cell lines by electroporation. BHK, C127 and COS cell lines were used for transient transfections of the plasmid. The transfection efficiency was monitored using a plasmid encoding green fluorescent protein – pEGFP and also a plasmid encoding β-galactosidase – pMV10. Transfected cells were subjected to immunofluorescence and western blot analysis in conjunction with control cells that received pcDNA3 alone and cells that did not receive DNA as negative controls. For immunofluorescence the primary antibody used was rabbit anti-gp150 antibody followed by swine anti-rabbit FITC conjugated secondary antibody.

Figure 3.5.5. Mammalian expression vectors pcDNA3 and pcDNA3.1



In order to generate a construct for expression of gp150 in eukaryotic cells the gene encoding gp150 was inserted into the multiple cloning site (MCS) of (**A**) pcDNA3 and (**B**) pcDNA3.1 between restriction sites *Hin*dIII and *Xho*I and *Hin*dIII and *Eco*RI respectively. This positions the gp150 gene under the control of the constitutive CMV promoter (P_{CMV}) and upstream of the bovine growth hormone polyadenylation signal (BGHpA). Other features inculde the ampicillin resistance gene (Ampicillin), f1 origin of replication (f1 ori), Simian virus 40 origin of replication (SV40 ori), neomycin resistance gene (Neomycin), SV40 polyadenylation signal (SV40pA), the bacterial origin of replication (ColE1) and the T7 and Sp6 promoters for *in vitro* transcription. Note: pcDNA3.1 is an updated version of pcDNA3 the main differences are the more extensive range of restriction sites in the MCS and the absence of the Sp6 promoter (Sp6).

By immunofluorescence no positive staining for gp150 could be observed for the pcDNA3/gp150 transfected cells at 24 and 48 hours post transfection, in contrast to MHV-68 infected cells used as a positive control for gp150 expression. Likewise, gp150 expression was not detected by western blot analysis. The standard quantity of plasmid DNA used for transfection was 30μg. In view of the absence of expression, transfection with 10μg and 100μg of plasmid DNA was tested however, gp150 protein detection was negative. In the event that the large DNA preparation of pcDNA/gp150 was in someway toxic to the cells a fresh DNA preparation was generated by the alternative use of a commercial kit. Transfection of mammalian cells with this fresh preparation did not result in detection of gp150 expression. In summary detection at the protein level was negative for gp150. Sequencing analysis of the entire gp150 insert in the plasmid preparations using the sequencing primers displayed in section 2.1.8 showed the gp150 insert to be 100% intact therefore lack of expression did not appear to be due to mutation of the plasmid insert.

3.4.1 PcDNA3.1/gp150

In light of the lack of detectable protein expression, despite the inserted sequence 100% intact, efforts were made to generate a new construct using an updated version of pcDNA3 known as pcDNA3.1. PcDNA3.1 is essentially the same as pcDNA3 the two main differences are the absence of the bacteriophage SP6 promoter site and the presence of a multiple cloning region with a more extensive range of restriction enzyme cleavage sites (refer to figures 3.5.5a & b). According to the manufacturers (Invitrogen) the SP6 promoter site, which is positioned downstream of the site for insertion of genes for expression, has been removed as it is thought to cause secondary structures to occur in RNA transcripts which are detrimental to protein expression.

In this instance gp150 was amplified from the MHV-68 genome using primers IA3 and IA4 (refer to section 2.1.8 for primers and PCR conditions). At the time of cloning the product of IA3 and IA4 – essentially the same as that of IE1gp150 and IE2gp150 but with alternative restriction sites, was immediately available so this was

used instead. IA3 includes the translational start site of gp150 and the nucleotides directly upstream i.e. the Kozak sequence is that present in the viral genome. The product of IA3 and IA4 was cut with *Hin*dIII and *Eco*RI, purified and ligated into pcDNA3.1 (previously prepared by digestion with *Hin*dIII and *Eco*RI followed by dephoshorylation). The ligation mix was used to transform competent XL1-Blue *E. coli* by the heat shock method. Of the 18 colonies that grew, which were noticeably smaller than bacteria transformed with pcDNA3.1 alone (no insert), 12 were grown up in liquid culture and subjected to DNA extraction. Restriction digestion of the plasmid DNA with *Hin*dIII and *Eco*RI showed that all contained the gp150 insert with the appearance of two bands – one of approximately 1.5kbp and another of 5.4kbp. One bacterial clone was grown up and a large plasmid preparation prepared. This pcDNA3.1/gp150 construct was transfected into COS, BHK and C127 cells and expression of gp150 was assessed by immunofluorescence and western blotting of cells. Protein expression of gp150 was not detected at 24 or 48 hours post transfection of cells.

Sequencing analysis (using the primers shown in section 2.1.8) showed the gp150 gene insert in the pcDNA3.1/gp150 construct to be 100% intact. In addition, restriction digests of pcDNA3/gp150 and pcDNA3.1/gp150 were done to confirm the identity of the plasmid backbone. At the time of cloning several other plasmids produced by Invitrogen i.e. pIND/Hygro and pIND(SP1)/Hygro were in use, these have a very similar size (less than 200bp difference) to pcDNA3 therefore confirmation of the correct plasmid was of heightened importance. For diagnostic restriction digestion *Nco*I was chosen due to differing site number between the plasmids (2 sites in pcDNA3/gp150 and pcDNA3.1/gp150, three sites in pIND/Hygro/gp150 and pIND(SP1)/Hygro/gp150). The backbone identity of pcDNA3/gp150 and pcDNA3.1/gp150 was correct.

3.4.2 pVR1255/gp150

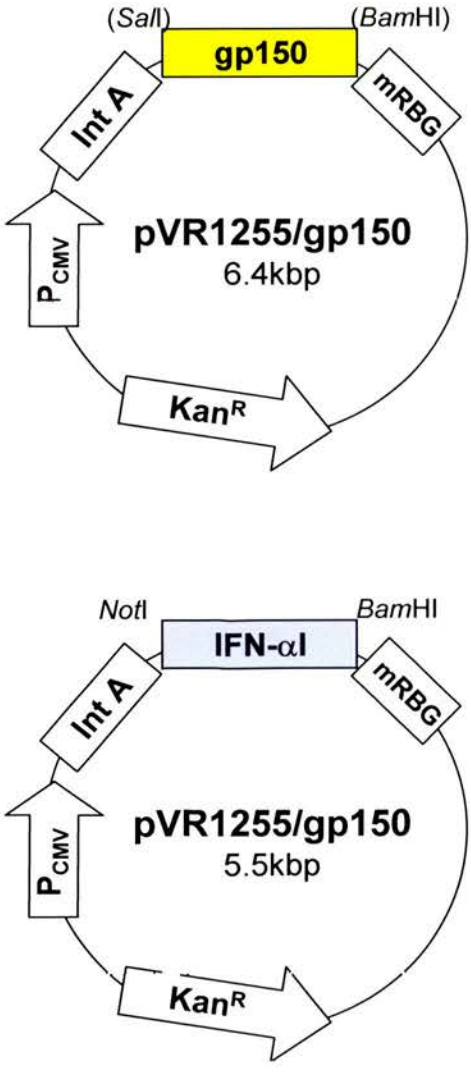
As part of a collaborative effort, Filippo Belardelli, Instituto Superiore di Sanità, Rome, was supplied with pSP72/gp150, this plasmid contains the entire cDNA for gp150. From pSP72/gp150 his laboratory cloned the gp150 gene and inserted this into the mammalian expression vector pVR1255 (Hartikka *et al.*, 1996), between restriction sites *Sal*I and *Bam*HI, downstream of the CMV immediate early promoter (refer to figure 3.5.6). The construct, named pVR1255/gp150 was supplied to our laboratory to be tested for expression of gp150 when transfected into mammalian cells. C127 murine epithelial cells were transfected with pVR1255/gp150 by electroporation. As figure 3.5.7 shows expression of gp150 was successfully detected 24 hours post transfection by immunofluorescence using anti-gp150 antibody. Western blot analysis was also done and the characteristic gp150 banding pattern was seen with bands detected at 110, 130 and 150kDa when probed with anti-gp150 antibody corresponding with those detected by Stewart *et al.*, 1996.

3.4.3 Generation of pVR1255/gp150-coated Microparticles for DNA Vaccination

With confirmation of mammalian expression of gp150 the pVR1255/gp150 plasmid was considered suitable for use in DNA vaccination studies. A large DNA preparation of endotoxin free pVR1255/gp150 was supplied by Filippo Belardelli, Instituto Superiore di Sanità, Rome. This was used to coat 1.6 μm gold particles for the generation of pVR1255/gp150 charged bullets required for administration of DNA to animals using the Helios gene gun system.

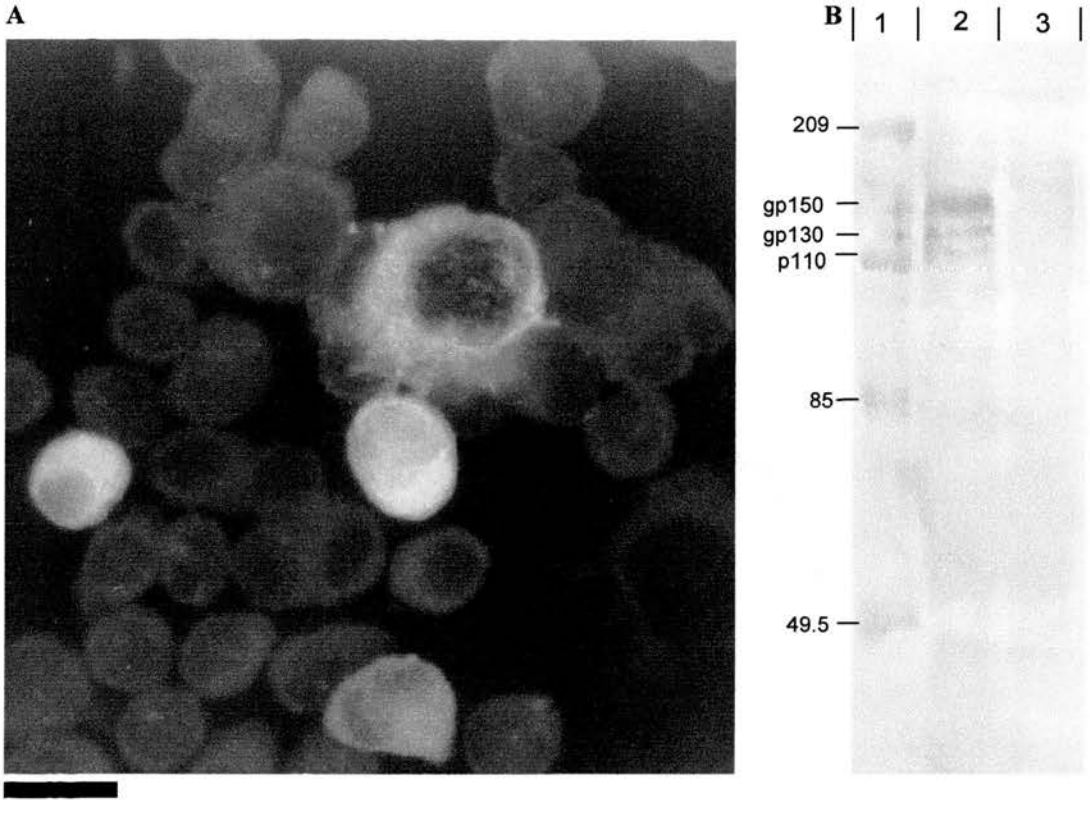
Production of the gene gun 'bullets' includes precipitating the chosen DNA onto gold particles which are then used to coat the inside of a tube, sections of the tube constitute the gene gun 'bullets'. Even coating of the tube is desired however, this can be difficult to achieve. In regard to this the level of polyvinyl propylene (PVP) in the DNA/gold particle preparation is of importance therefore different concentrations were tested 0.01mg/ml, 0.05mg/m and a preparation with no PVP. The best coating was achieved using 0.01mg/ml and therefore this level was used for all further preparations.

Figure 3.5.6. Vectors pVR1255/gp150 & pVR1255/IFN-αI for mammalian expression of gp150 and interferon alpha I



For mammalian expression of gp150 and interferon alpha I vectors pVR1255/gp150 and pVR1255/IFNαI were used, respectively. The gp150 and interferon alpha I (IFN-αI) genes for expression are under the control of the CMV immediate early promoter (P_{CMV}) with the CMV intron A (Int A) postioned between promoter and gene to enhance expression. Downstream of the inserted genes is the minimal rabbit β globin terminator (mRBG) and plasmid carries the kanamycin resistance gene (Kan^R). The gp150 gene for expression was inserted into pVR1255 between restriction sites *Sal*I and *Bam*HI these are indicated in brackets as the inserts had *Xho*I and *Bgl*II cut ends therefore the restriction sites were eliminated upon ligation. These plasmids were supplied by Dr. F. Belardelli.

Figure 3.5.7. Expression of gp150 by C127 murine epithelial cells transfected with pVR1255/gp150



A. Immunofluorescence detection of gp150 expression by C127 murine epithelial cells transfected with pVR1255/gp150, 48 hours post transfection by electroporation. Cells were acetone fixed and then probed with rabbit anti-gp150 antibody, followed by a swine anti-rabbit fluoresceine isothiocyanate (FITC) conjugated antibody. Fluorescence was visualised by ultra violet microscopy. Size bar represents 10μm. **B.** Western blot of C127 cells 48 hours post transfection with pVR1255/gp150 (lane 2). Cell lysates were electrophoresed through a 7.5% acrylamide SDS-PAGE gel and transferred to an immobulin-P membrane. The blot was probed with rabbit anti-gp150 antibody followed by a swine anti-rabbit alkaline phosphatase conjugated antibody and subsequent addition of the alkaline phosphatase substrate NBT/BCIP (nitro blue tetrazonium/5-bromo-4-chloro-3-indoyl phosphate). Lane 1 - MW marker, numbers to the left indicate the sizes in thousands. Lane 3 - normal C127 cells. Also indicated are the positions of the predominant bands gp150, gp130 and p110 which correlate with Stewart *et al.*, 1996.

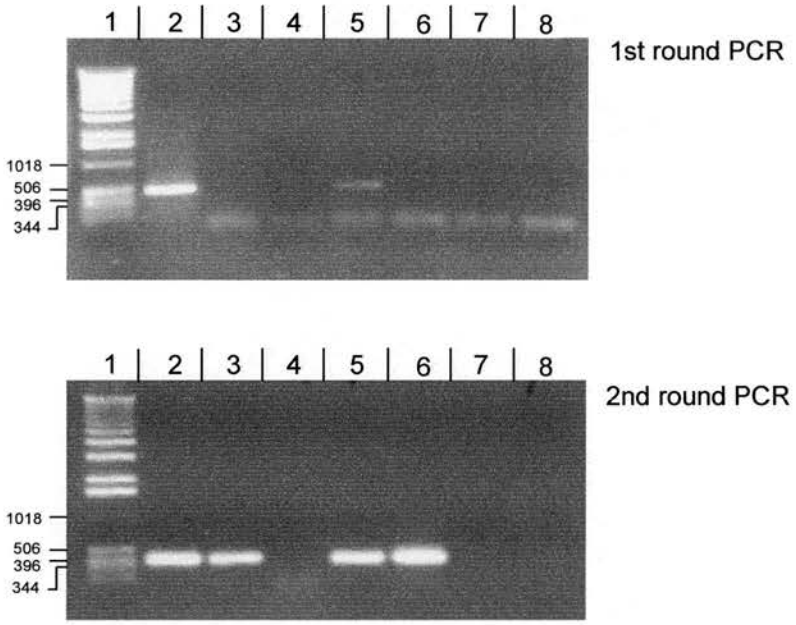
Alongside pVR1255/gp150 a large DNA plasmid preparation of endotoxin free pVR1255/IFN-αI was used to prepare pVR1255/IFN-αI charged gene gun bullets. pVR1255/IFN-αI consists of the mammalian expression vector pVR1255 with the gene encoding murine interferon alpha I (IFN-αI) inserted between sites *Bam*HI and *Nco*I of the multiple cloning site (refer to figure 3.5.6). This plasmid was supplied by Felippo Belardelli. Expression IFN-αI from pVR1255/IFN-αI was confirmed by the Belardelli laboratory using a vesicular stomatitis virus (VSV) growth inhibition assay (Familletti *et al.*, 1981, Vogel *et al.*, 1991).

3.4.4 Testing Gp150 Expression from pVR1255/gp150 Gene Gun Bullets

The pVR1255/gp150 gene gun bullets were tested for expression *in vitro* and *in vivo*, to ensure the DNA, following incorporation into bullets, was functional. C127 epithelial cell monolayers in six well plates were bombarded with the pVR1255/gp150 microparticles and the expression of gp150 mRNA was analysed by RT-PCR 24 hours post transfection. As figure 3.5.8 shows, at 24 hours post transfection, a band of approximately 368bp was generated following nested RT-PCR using primers PAG1 and PAG2 for 40 cycles and internal primers PAG11 and 12 for a subsequent 25 cycles (for primers and PCR conditions see section 2.1.8).

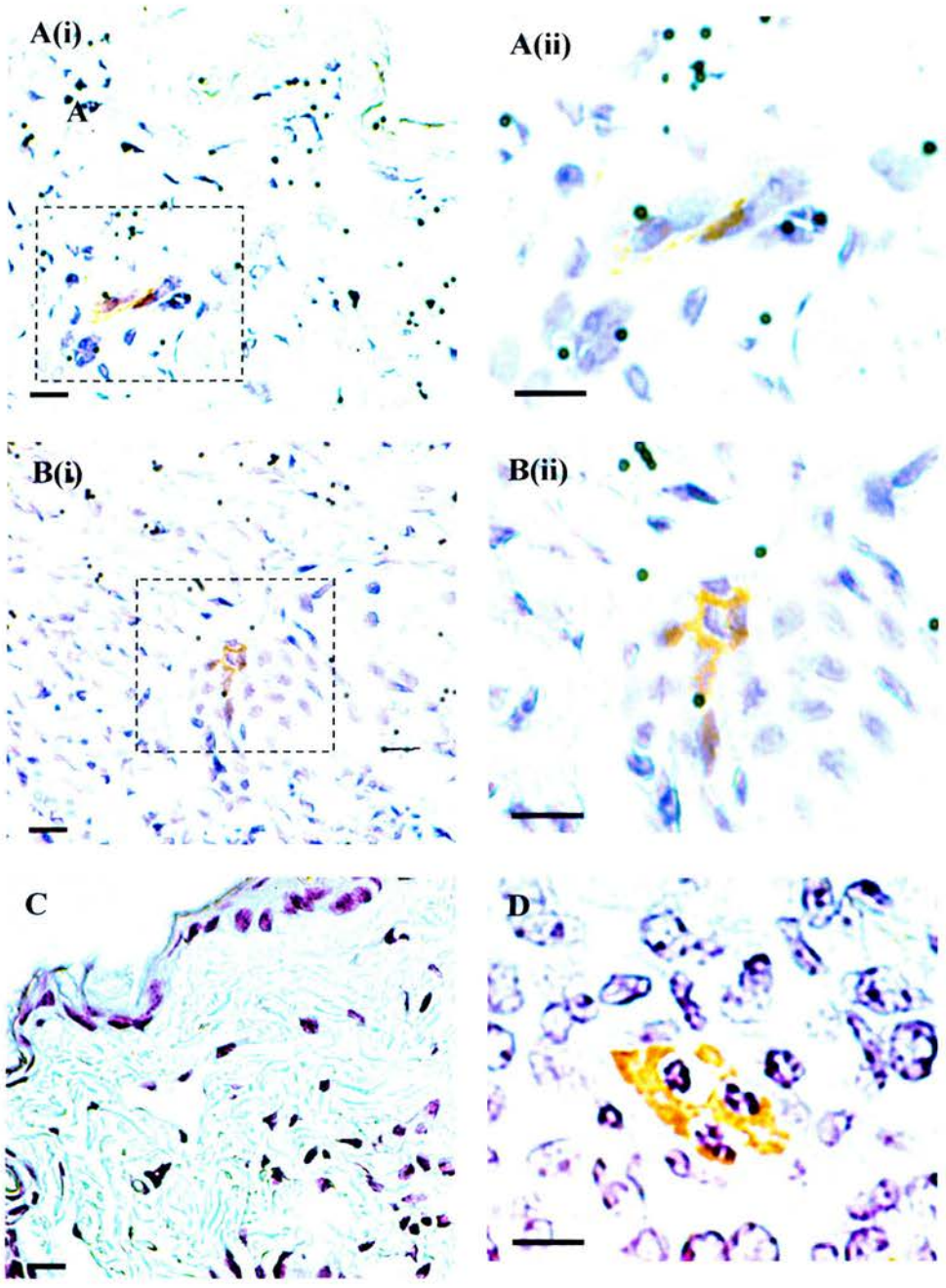
Gold particles coated with pVR1255/gp150 were administered to the abdominal region of C57BL/6 mice using the Helios gene gun system at a helium pressure of 500psi. Sectionsof 6μm thickness were cut from formalin fixed paraffin-embedded abdominal skin biopsies taken 2, 4 and 24 hours post DNA administration. As figure 3.5.9 shows gp150 expression was detected in the dermis at 24 hours post DNA administration using anti-gp150 antibody to immunostain sections. Gp150 expression was not detected in sections of biopsies taken at earlier times post transfection.

Figure 3.5.8. Nested RT-PCR analysis of expression of gp150 mRNA in cells transfected with pVR1255/gp150 using the Helios gene gun system



pVR1255/gp150 gene gun bullets were tested for expression of gp150 *in vitro*, to ensure the DNA, following incorporation into bullets, was functional. C127 epithelial cell monolayers in six well plates were bombarded with pVR1255/gp150 coated gold microparticles using the Helios gene gun at a helium pressure of 50psi. The expression of gp150 mRNA was analysed by nested RT-PCR 24 hours post transfection. Following isolation of mRNA and reverse transcription (RT-) two rounds of polymerase chain reaction (PCR) were done. The first round of PCR was done using primers PAG1 & 2 for 40 cycles (expected product 501bp). The second round of PCR was done on 10µl of product from the first amplification using internal primers PAG11 & PAG12 for a subsequent 25 cycles (expected product 368bp). Products were analysed on 0.7% agarose gels containing ethidium bromide and visualised using a UV transilluminator. Lanes: 1- 1 kb DNA ladder marker, size of pertinent bands are shown at the left 2 - MHV-68 DNA, positive PCR control 3 - mRNA from MHV-68 infected cells 4 - untransfected cells, negative control, 5 & 6 - pVR1255/gp150 transfected cells, 7 & 8 - pVR1255/gp150 transfected cells not reverse transcribed, control for contaminating DNA.

Figure 3.5.9. Expression of gp150 *in vivo* following gene gun administration of pVR1255/gp150 into the abdominal dermis of C57BL/6 mice



To check for expression of gp150 *in vivo* pVR1255/gp150 was administered to the abdomen of C57BL/6 mice by microparticle bombardment using the Helios gene gun system. Sections of 6μm thickness were cut from formalin fixed paraffin-embedded skin biopsies taken 24 hours post bombardment from the gene gun targetted abdomen region (A & B). Negative control sections were derived from unadministered abdominal skin (C). Positive control tissue for gp150 detection by immunostaining was derived from a S11 tumour biopsy from a BALB/c nude mouse (D). Immunostaining was performed to detect the presence of gp150, rabbit anti-gp150 antibody was used followed by a goat anti-rabbit biotinylated antibody and DAB staining. Slides were negative as in (C) when use of normal rabbit serum, in place of rabbit anti-gp150 antibody to control for gp150 specific staining, was performed. Boxes in panels A(i) and B(ii) indicate the magnified areas shown in A(ii) and B(ii) respectively. The black bars represent 10μm across.

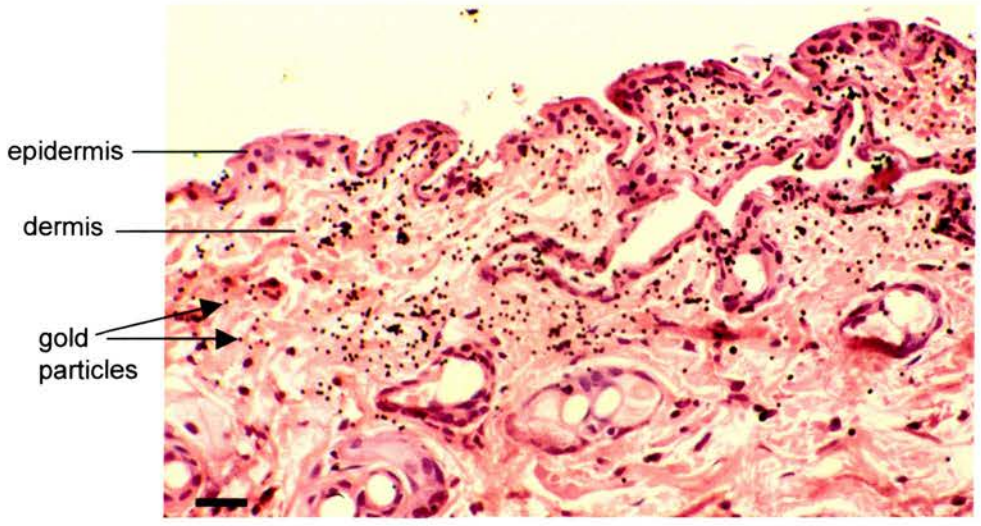
3.4.5 Optimum Pressure for Delivery of DNA Coated Gold Particles into the Dermis

The optimum helium pressure for gene gun inoculation of the abdominal tissue of mice was tested. The aim was to ensure gold particle DNA delivery into the dermis of the skin as this is highly populated with the dendritic Langerhans cells making the area highly immunologically responsive. Following administration of gold particles at different pressures (400psi, 500psi and 600psi) biopsies of the targetted abdominal skin were taken. Six μm sections of the biopsies embedded in paraffin were cut onto slides and haematoxylin and eosin stained. Of the pressures tested 500psi was selected for further use as examination of sections by microscopy showed this pressure resulted in the delivery of substantial numbers of gold particles in the desired region (refer to figure 3.6.1). In comparison, 400psi and 600psi produced good levels of gold particles in the dermis but over all the sections studied (nine for each pressure) 500psi more consistently produced location of particles in this area.

3.4.6 Vaccinia Recombinant Viruses – VV^{gp150} & VV^{gpt}

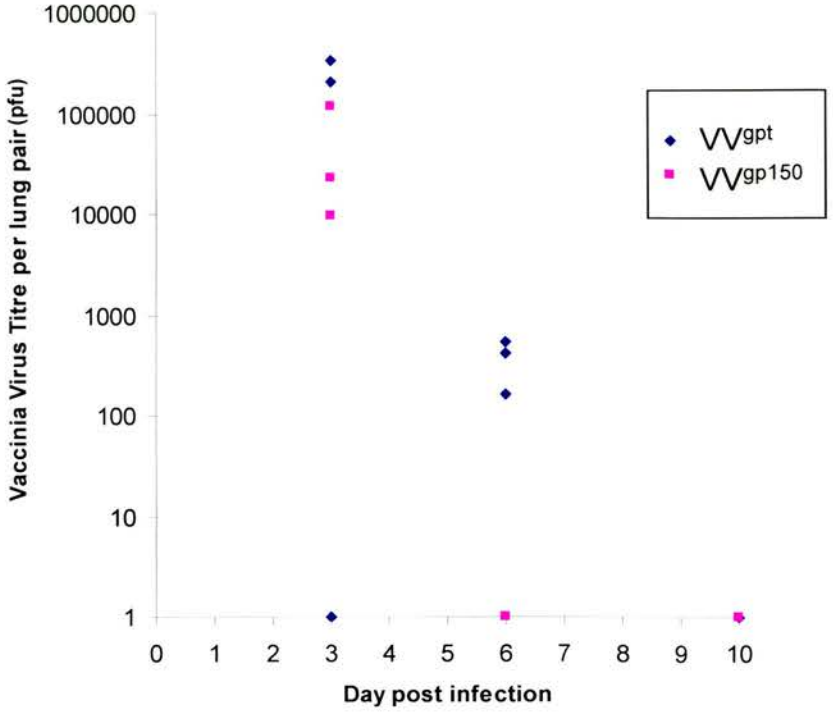
A recombinant vaccinia virus VV^{gp150} (Stewart *et al.*, 1999a) containing the gp150 encoding gene inserted into the thymidine kinase (*tk*) locus under the control of the vaccinia 7.5K early/late promoter and the *E. coli* guanosine phosphoribosyl transferase gene (*gpt*) as a selectable marker was supplied by Dr.J.P Stewart. A control recombinant vaccinia virus VV^{gpt}, with *gpt* alone (i.e. no additional foreign gene) was also made available (Stewart *et al.*, 1999a). These recombinant vaccinia viruses were generated and used in a previous study which showed VV^{gp150} to specifically induce MHV-68 neutralising antibodies when introduced, at a dose of 10^7 pfu, into BALB/c mice sub-cutaneously. Stocks of these viruses were grown up ready for use. Prior to use in the vaccination trial a preliminary *in vivo* assay was done to ensure the virus preparations were not toxic and to test the lung viral titres following intranasal infection. C57BL/6 mice were infected with 2×10^6 pfu by the intranasal route, on days 3, 6 and 10 post infection the lungs were removed and the virus titre analysed by conventional plaque assay. As figure 3.6.2 shows both vaccinia recombinants were cleared from the lungs by day 10 post infection. A higher titre of VV^{gpt}, (mean virus titre 181,200 pfu) approximately 3.5 times greater,

Figure 3.6.1. Gold particle localisation in the abdominal dermis of C57BL/6 mice following administration using the Helios gene gun



A pressure of 500psi was used to bombard the abdomen of C57BL/6 mice with 1.6μm gold particles using the Helios gene gun system in order to obtain dermal localisation of particles. Skin biopsies were taken immediately following administration of particles and fixed in formaldehyde. Biopsies were embedded in parraffin, cut into 6 μm sections and hematoxylin and eosin stained. Light microscopy was used to examine the tissues sections. The gold particles are seen here as black dots. The size bar represents 20μm across.

Figure 3.6.2. Vaccinia virus titre in lungs of C57BL/6 mice



C57BL/6 mice, approximately six weeks old, were infected intranasally with 2 x 10^6 pfu (50µl) of either a vaccinia virus recombinant containing the *E.coli* gene for guanosine phosphoribosyl transferase (VV^{gpt}) or a vaccinia virus recombinant containing the gene for gp150 and *gpt* of MHV-68 (VV^{gp150}). Lungs were taken from three mice per group at each time point (days 3, 6 and 10 post infection). Virus titres of lung homogenates were determined by conventional plaque assay on C127 epithelial cell monolayers in duplicates. pfu = plaque forming units.

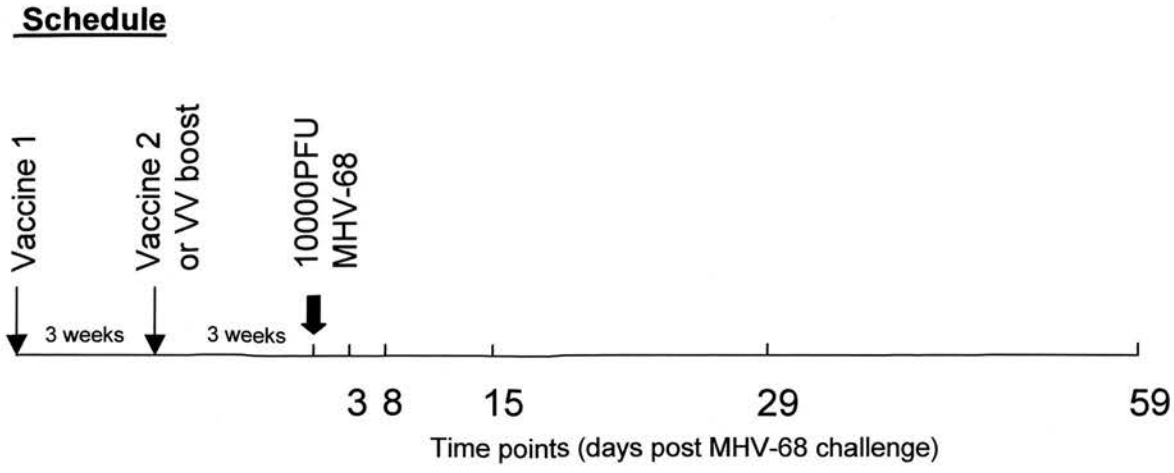
compared to VV^{gp150} (mean virus titre 50, 466 pfu) was detected at day 3 post infection. VV^{gp150} was cleared from the lungs by day 6 post infection whereas approximately 500 pfu of VV^{gpt} could be detected at this stage.

3.4.7 Gp150 DNA & Vaccinia Virus Vaccination Trial

In order to determine the efficacy of a gp150 DNA vaccine to protect against MHV-68 infection individual groups of C57BL/6 mice were subject to different vaccine regimens. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) alone or in conjunction with pVR1255/IFN-αI (IFN-αI DNA) followed by a booster three weeks later of either gp150 DNA, Gp150 DNA plus IFN-αI DNA or the gp150 recombinant vaccinia virus VV^{gp150}. The vaccine schedule for each group is summarised in figure 3.6.3. Control groups included mice that received no vaccine, gp150 DNA in conjunction with the *gpt* recombinant vaccinia virus (VV^{gpt}) and mice that received IFN-αI DNA alone. A control plasmid lacking an insert i.e. pVR1255 was not available for inclusion in this experiment. The C57BL/6 strain of mice was selected due to the known susceptibility to MHV-68 and to maintain continuity with previous studies. These studies include the immunisation of C57BL/6 mice using dendritic cells pulsed with MHV-68 lytic cycle T cell epitopes (Liu *et al.*, 1999) and the identification of MHC class I-restricted epitopes that drive the primary $CD8^+$ T cell response to MHV-68 infection in C57BL/6 (Stevenson *et al.*, 1999).

Plasmid DNA was administered to the abdomen of mice using the gene gun. One shot (1μg DNA) was used per plasmid for each vaccination. Three weeks following the primary immunisation with DNA the second immunisation/boost with either DNA or vaccinia virus was done. Recombinant vaccinia virus, 2×10^6 pfu, was administered intranasally. This dose was selected on the basis of previous work using a recombinant vaccinia virus to induce lung antibody against RSV glycoprotein G (Stott *et al.*, 1987). Intranasal challenge was used, as opposed to intramuscular (i.m.) or intraperitoneal (i.p.) routes, in order to target mucosal immunity. Three weeks after the second vaccination all groups were challenged with 1×10^4 pfu of MHV-68 per animal by the intranasal route. This challenge dose is lower than the

Figure 3.6.3 Gp150 DNA vaccination schedule & immunisation regimens



Groups

Group 1 - MHV-68 Only

Group 2 - 2x [gp150 DNA] + MHV-68

Group 3 - 1x [gp150 DNA], 1x VV^{gp150}(i.n.) + MHV-68

Group 4 - 1x [gp150 DNA], 1x VV^{gpt} (i.n.) + MHV-68

Group 5 - 2x [gp150 DNA + IFN-α1 DNA] + MHV-68

Group 6 - 2x [IFN-α1 DNA] + MHV-68

Data Analysed

Serum antibody measured by ELISA

Lung titre

Infectious centres in spleen

Splenomegaly

Six groups of C57BL/6 mice, approximately 8 weeks of age, received combinations of vaccines as indicated. Vaccine components were: pVR1255/gp150 plasmid DNA (gp150 DNA), pVR1255/IFN-αI plasmid DNA (IFN αI DNA), and recombinat vaccinia viruses VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E. coli* guanosine phosphoribosyl transferase gene (*gpt*), the control recombinant virus - VV^{gpt}, contains *gpt* alone. Vaccine regimens included priming animals with gp150 DNA alone or in conjunction with IFN-αI DNA (group 5) followed by a booster three weeks later of either gp150 DNA alone (group 2), Gp150 DNA + IFN-αI DNA (group 5) or one of two recombinant vaccinia viruses - VV^{gp150} (group 3) and VV^{gpt} (group 4). Group 1 were an unvaccinated control and group 6 received IFN-αI alone at both vaccine administrations. At each vaccine administration (vaccine 1 and vaccine 2) the DNA was delivered to the abdominal dermis of mice using a Helios gene gun, one shot (1μg DNA) per plasmid. Intranasal (i.n.) administration of 2×10^6 pfu of recombinant vaccinia virus per animal was done. Three weeks after the second immunisation the mice were subject to intranasal challenge with 1×10^4 pfu of MHV-68. Four mice per group were taken at each time point for analysis of MHV-68 infectious lung titre, MHV-68 infectious centres of the spleen (latent MHV-68), extent of splenomegaly and serological response to MHV-68.

standard dose of 4 x 10^5 pfu routinely used by this laboratory and was selected in order to promote the detection of immune control of the virus. Mice challenged with 1 x 10^4 pfu MHV-68 undergo the same course of infection resulting from the standard challenge dose albeit peak lung virus titre and presence of latent virus in the spleen is slightly delayed (unpublished observations, Phillipo/Allen).

Four mice per group were taken at each time point post MHV-68 challenge (days 3, 8, 15, 29 and 59) in order to analyse the MHV-68 infectious lung titre, infectious centre titre of the spleen, extent of splenomegaly and the serological response to MHV-68.

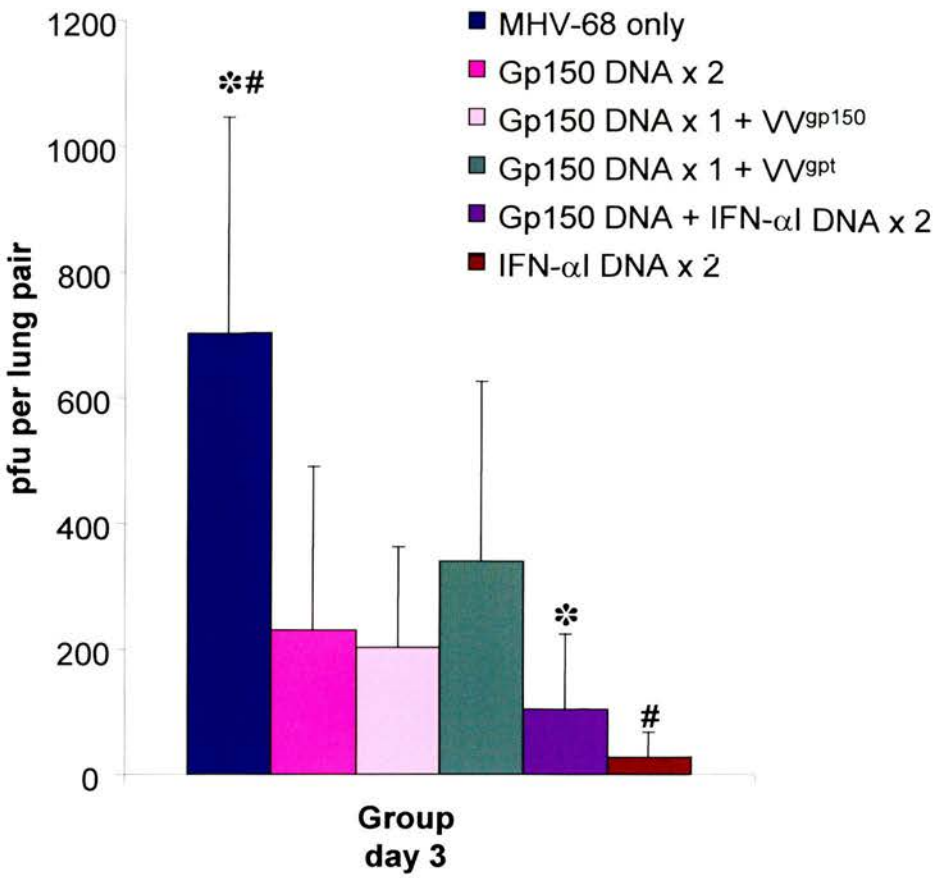
3.4.8 Effect of Vaccination on Viral Lung Titre

To assess the effect of the vaccine regimens on the acute phase of MHV-68 infection infectious lung titres were analysed by conventional plaque assay. For each group infectious virus was detected in the lung at day 3 post infection alone, all later time points were negative for infectious virus. As figure 3.6.4a shows at day 3 post infection control mice, which did not receive any form of vaccination, had a mean MHV-68 lung titre of approximately 700 pfu. In comparison, the other groups show a trend towards a reduction in infectious lung titre (ranging from 25-350pfu/lung) however, by the Student's t test only groups 5 and 6 (those that received gp150 DNA + IFN-αI DNA and IFN-αI DNA alone) are statistically different by Student's t test with P values of 0.046 and 0.03 respectively. Figure 3.6.4b shows the day 3 lung titre data for each animal to provide an indication of the spread of the data.

3.4.9 Effect of Vaccination on Splenomegaly and Virus Latency in the Spleen

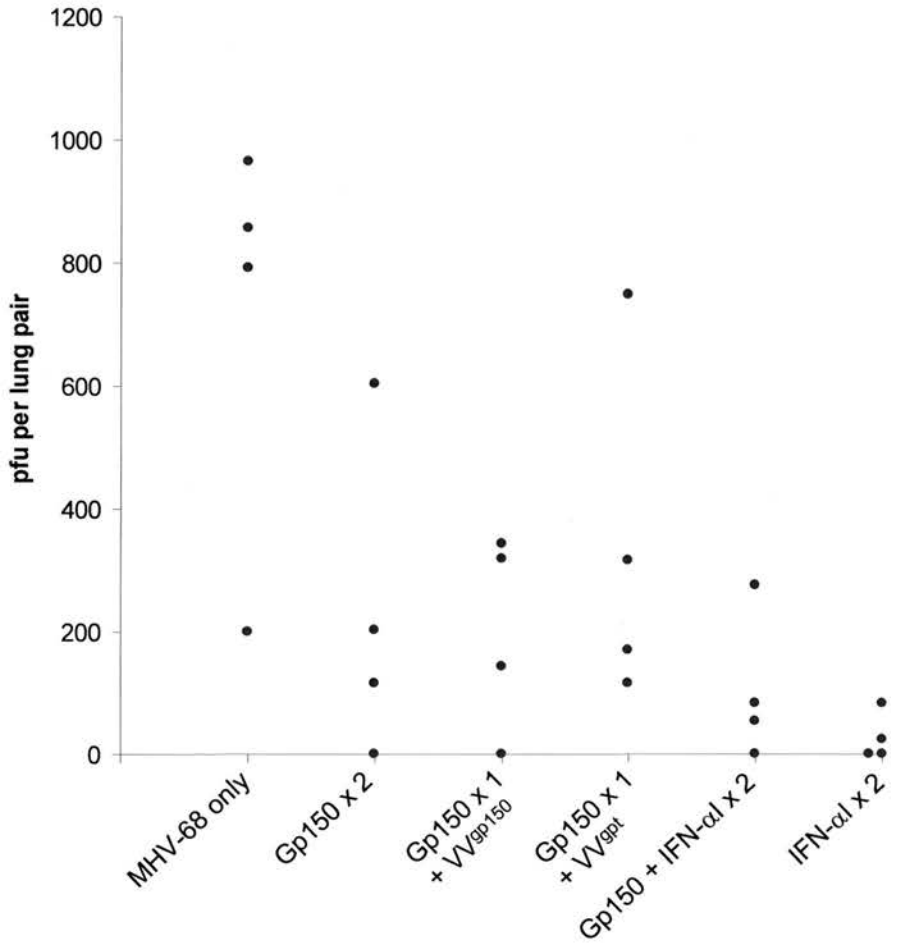
Following acute infection of the lung MHV-68 is known to establish latency in B cells, lung epithelium and macrophages (Stewart *et al.*, 1998, Sunil-Chandra *et al.*, 1992b, Weck *et al.*, 1999). The number of latently infected cells reaches a peak level, of approximately 1:10^4 spleen cells, two weeks after infection and declines to a stable level of approximately 1:10^6 spleen cells by day 21 (Sunil-Chandra *et al.*, 1992a, Cardin *et al.*, 1996). The establishment of latency is characterised by transient splenomegaly (Sunil-Chandra *et al.*, 1992a). To assess the effect of MHV-68 on the

Figure 3.6.4a Lung virus titres on day 3 post MHV-68 infection in C57BL/6 mice vaccinated with different regimens



C57BL/6 mice were vaccinated with the indicated regimens and challenged intranasally with 1 x 10^4 pfu of MHV-68 21 days following the final administration of vaccine. The MHV-68 lung titre was determined by conventional plaque assay. Only day 3 post MHV-68 infection (time points tested included day 3, 8, 15, 29 and 59) yielded virus for each group. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) alone or in conjunction with pVR1255/IFN-αI (IFN- αI DNA) followed by a booster three weeks later of either gp150 DNA, Gp150 DNA + IFN -αI DNA or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control, VV^{gpt}, contains *gpt* alone. Plasmids were administered using gene gun delivery to the abdomen of mice, one shot (1μg DNA) per plasmid. Intranasal administration of 2 x 10^6 pfu recombinant vaccinia virus was done. The data represent the mean values from four mice, bars denote the standard deviation. The limit of detection was 10 pfu (plaque forming units) per lung. Statistical difference was calculated using Student's t test.

Figure 3.6.4b Lung virus titres on day 3 post MHV-68 infection in C57BL/6 mice vaccinated with different regimens



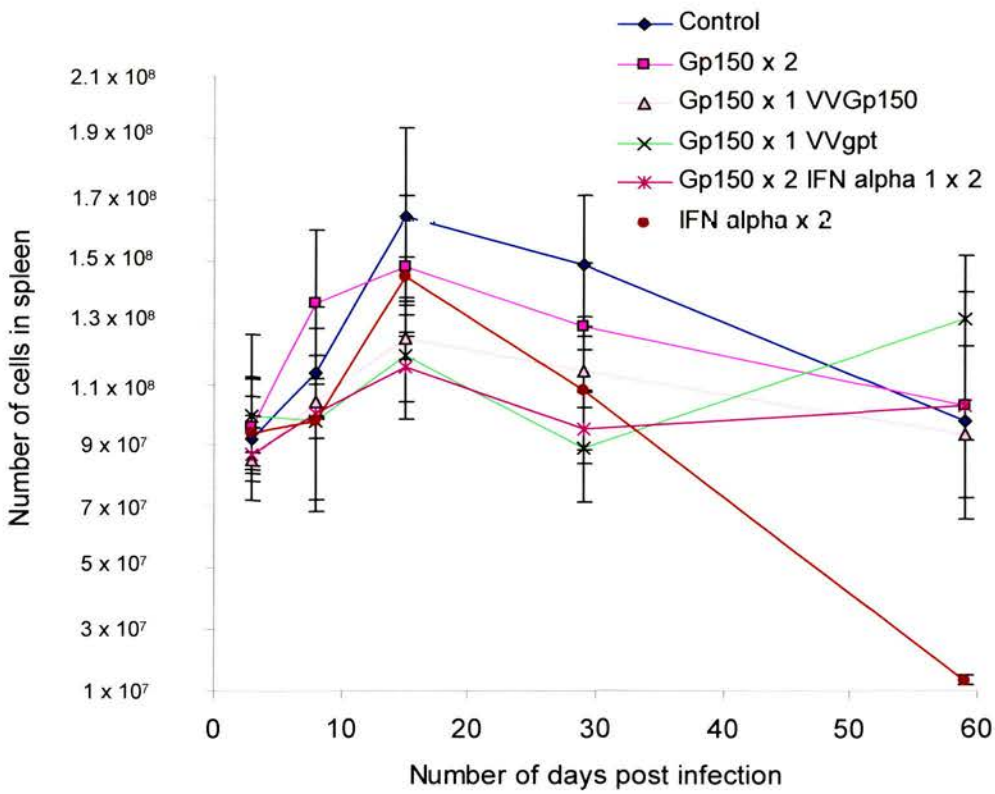
C57BL/6 mice were vaccinated with the indicated regimens and challenged intranasally with 1 x 10^4 pfu of MHV-68 21 days following the final administration of vaccine. The MHV-68 lung titre was determined by conventional plaque assay. Only day 3 post MHV-68 infection (time points tested included day 3, 8, 15, 29 and 59) yielded virus for each group. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) alone or in conjunction with pVR1255/IFN-αI (IFN- αI DNA) followed by a booster three weeks later of either gp150 DNA, Gp150 DNA + IFN -αI DNA or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control, VV^{gpt}, contains *gpt* alone. Plasmids were administered using gene gun delivery to the abdomen of mice, one shot (1μg DNA) per plasmid. Intranasal administration of 2 x 10^6 pfu recombinant vaccinia virus was done. The limit of detection was 10 pfu (plaque forming units) per lung. Statistical difference was calculated using Student's t test. The reduction in virus titres in the gp150 DNA + IFN- αI DNA and IFN- αI DNA x 2 were significantly different from the unvaccinated control animals as determined by a Student t test (P = 0.046 & 0.03, respectively).

lymphoid system and the ability of the virus to establish a latent infection in mice that had received different vaccination regimens spleen cell numbers were counted and the number of infectious centres in the spleen were titred at day 3, 8, 15, 29 and 59 for each group. Spleen cell numbers provide a measure of splenomegaly and infectious centre titres minus infectious virus titres determines the number of latently infected cells in the spleen.

As figure 3.6.5 shows, in the unvaccinated control group peak spleen cell numbers were detected at day 15 with levels approximately 1.8 times normal. At day 3, 8 and 15 spleen cell numbers in the other groups were not significantly different from the control however there appeared to be a trend towards lower cell numbers. At day 29 spleen cell numbers were declining, all groups except the group which received gp150 DNA alone had significantly lower levels compared to the control group by Student's t test ($P<0.05$). At day 59 spleen cell numbers had returned to approximately normal levels in all groups except the group which received IFN-αI DNA alone. This group was significantly different with approximately 10 times less spleen cells compared to the unvaccinated control.

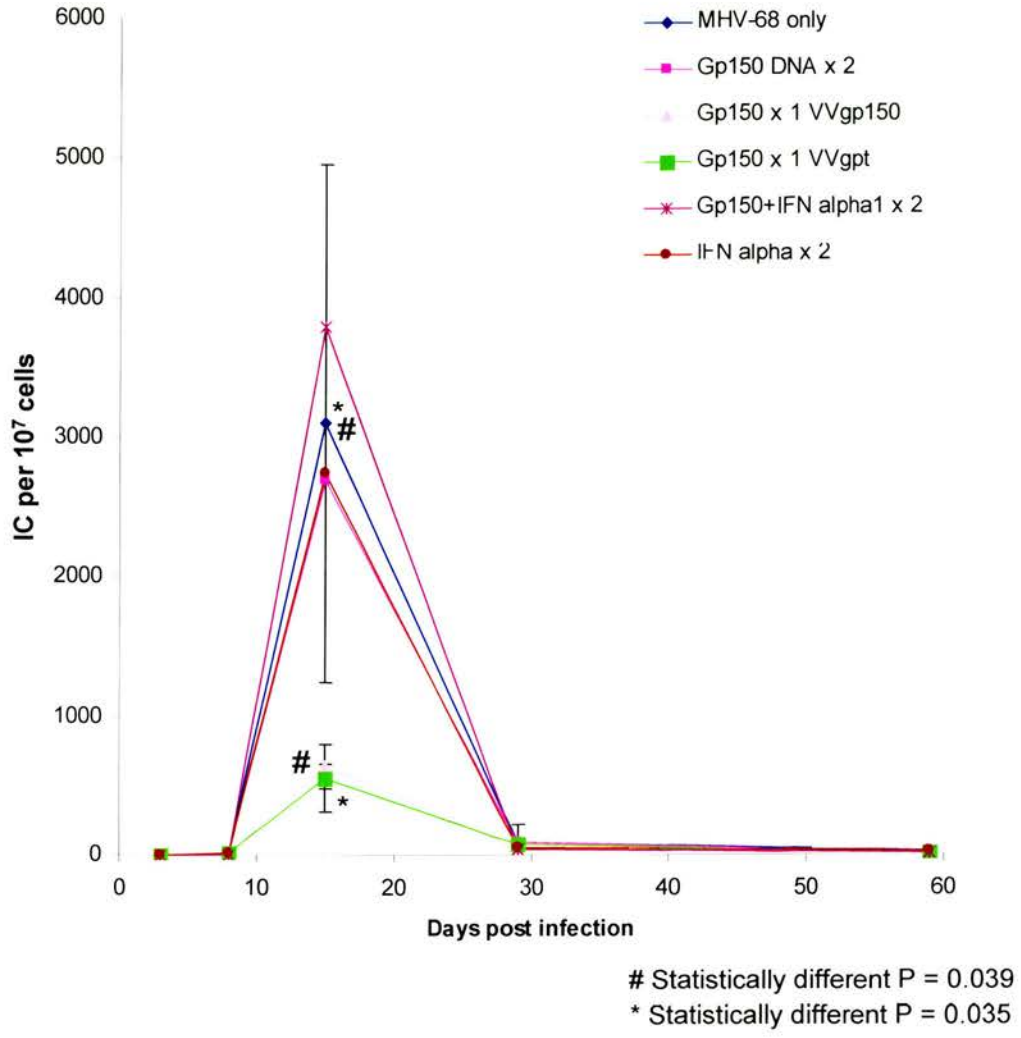
Infectious centre assays provided an indication of the level of MHV-68 latency in the spleen. As figure 3.6.6 shows the unvaccinated control followed the common pattern for MHV-68 infection with peak infectious centres, approximately $3:10^4$ spleen cells, detected at day 15. The mean number of infectious centres per spleen was approximately 56000. By day 59 infectious centre titre per spleen had resolved to a basal level (mean 230 infectious centres per spleen). All groups followed a similar trend infectious centre titres peaking at day 15 and then declining to a basal level by day 59. The groups which received gp150 DNA followed by a booster of either VV^{gp150} or VV^{gpt} had significantly lower peak infectious centre titres at day 15 post infection (by Student's t test gp150 + VV^{gp150} $P = 0.039$ and gp150 + VV^{gpt} $P = 0.035$). All the other groups were not significantly different from the control group at each time point. At day 29 and 59 the infectious centre levels of the groups which received recombinant vaccinia virus were not significantly different from the control group. Thus, although two groups had lower peak numbers of infectious centres

Figure 3.6.5. Spleen cell numbers in MHV-68 infected C57BL/6 mice vaccinated with different regimens



C57BL/6 mice were vaccinated with different regimens and challenged intranasally with 1 x 10^4 pfu of MHV-68 21 days following the final administration of vaccine. Data represent mean spleen cell numbers per spleen for four mice per group for each given time post intranasal challenge with MHV-68. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) alone or in conjunction with pVR1255/IFN-αI (IFN- αI DNA) followed by a booster three weeks later of either gp150 DNA, Gp150 DNA + IFN -αI DNA or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control, VV^{gpt}, contains *gpt* alone. Plasmids were administered using gene gun delivery to the abdomen of mice, one shot (1μg DNA) per plasmid. Intranasal administration of 2 x 10^6 pfu recombinant vaccinia virus was done. The control group received MHV-68 alone. The bars denote standard deviation.

Figure 3.6.6. Infectious centres in spleen post MHV-68 infection of C57BL/6 mice subject to different vaccine regimens



C57BL/6 mice were vaccinated with different regimens and challenged intranasally with 1 x 10^4 pfu of MHV-68 21 days following the final administration of vaccine. Data represent mean infectious centres (IC) per 10^7 splenocytes for four mice from each group. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) alone or in conjunction with pVR1255/IFN-αI (IFN- αI DNA) followed by a booster three weeks later of either gp150 DNA, Gp150 DNA + IFN -αI DNA or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control, VV^{gpt}, contains *gpt* alone. Plasmids were administered using gene gun delivery to the abdomen of mice, one shot (1μg DNA) per plasmid. Intranasal administration of 2 x 10^6 pfu recombinant vaccinia virus was done. The control group were unvaccinated and received MHV-68 alone. Bars denote standard deviation. Statistical significance was calculated using Student's t test.

ultimately the basal levels of latent virus in all groups at day 59 were not significantly different from the unvaccinated control. Infectious virus was detected only sporadically in the spleen of mice from each group at levels below 100pfu.

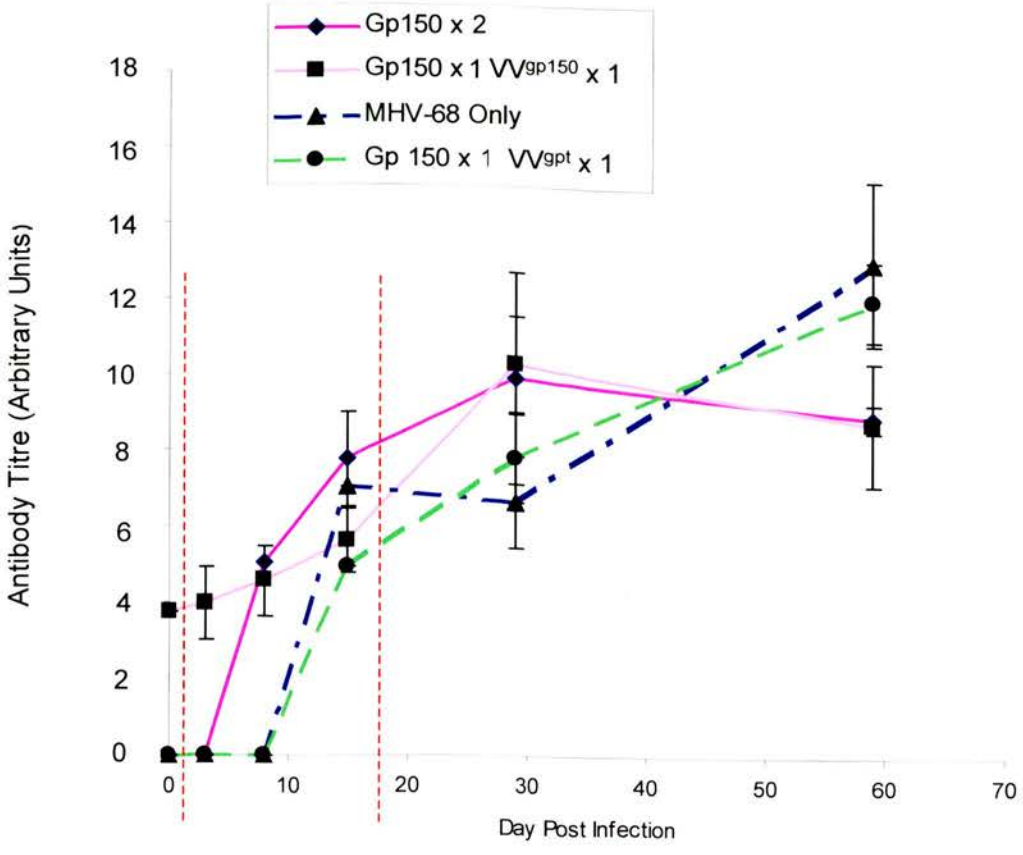
3.5.1 Effect of Vaccination on the Serological Response to MHV-68 Infection

Sera obtained from blood taken from mice prior to MHV-68 infection and day 3, 8, 15, 29 and 59 post MHV-68 challenge were analysed by ELISA to determine relative levels of MHV-68 specific antibody. Groups tested included the unvaccinated control, the recipients of gp150 DNA alone and gp150 DNA plus either VV^{gp150} or VV^{gpt}. As figure 3.6.7 shows MHV-68 specific antibody was not detected until day 15 post MHV-68 infection in the control group as was the case for those animals which received gp150 DNA followed by a booster of VV^{gpt}. In contrast, MHV-68 specific antibody was detected prior to infection in recipients of gp150 DNA plus VV^{gp150} and at day 8 post infection in recipients of gp150 DNA alone. By day 15 all groups tested had detectable MHV-68 specific antibody which continued to increase at day 29. By day 59 MHV-68 antibody levels appeared to be leveling off for the groups which received gp150 DNA alone or in conjunction with a recombinant vaccinia virus boost. The MHV-68 specific antibody levels for the unvaccinated control and the group receiving gp150 DNA in conjunction with the control vaccinia virus appeared to be on the incline upwards at day 59. From day 15 onwards the antibody levels of each group were not statistically different from the control group by the Student's t test.

Following on from the determination of the levels of MHV-68 specific antibody the immunoglobulin isotypes within the anti-MHV-68 antibody population were analysed from groups at selected time points. Time points for analysis were selected between day 3 and day 15 post infection (illustrated in figure 3.6.7 by red dotted lines) on the basis that MHV-68 specific antibody had been detected. Antibodies used for detection of different mouse immunoglobulin subclasses included anti IgG/A/M antibody, anti-IgG2a, anti-IgG2b, and an anti-IgG1 antibody. An anti-IgA antibody was not available. Figure 3.6.8 shows that the anti-MHV-68 antibody response detected in all groups was predominantly IgG2a and IgG2b – indicating a T

helper 1 (Th1) response. No antibody to MHV-68 was detected in the IgG1 or IgM subclass.

Figure 3.6.7. Titration of MHV-68 specific antibody reponse of C57BL/6 mice, vaccinated with different regimens, following MHV-68 infection



Sera was prepared from blood taken from C57BL/6 mice that had been vaccinated with different gp150 based regimens pre and post infection with MHV-68 (days 3, 8, 15, 29 and 59). An enzyme linked immunorbent assay was used to determine the levels of MHV-68 specific antibody in samples relative to a standard curve generated using dilutions of mouse anti-MHV-68 hyperimmune antiserum. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) followed by a booster three weeks later of either gp150 DNA, or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control virus VV^{gpt} contains *gpt* alone. Plasmid was administered using gene gun delivery to the abdomen of mice, one shot (1µg DNA) per plasmid. Intranasal administration of 2×10^6 pfu recombinant vaccinia virus was done. The unvaccinated control group received MHV-68 alone. Data represent mean values from four mice, bars indicate standard deviation. The data points positive for anti-MHV-68 specific antibody within the red dotted lines were selected for analysis of immunoglobulin isotype populations.

Figure 3.6.8. Analysis of immunoglobulin subclass of the MHV-68 specific antibody response post MHV-68 challenge in C57BL/6 mice vaccinated with different regimens

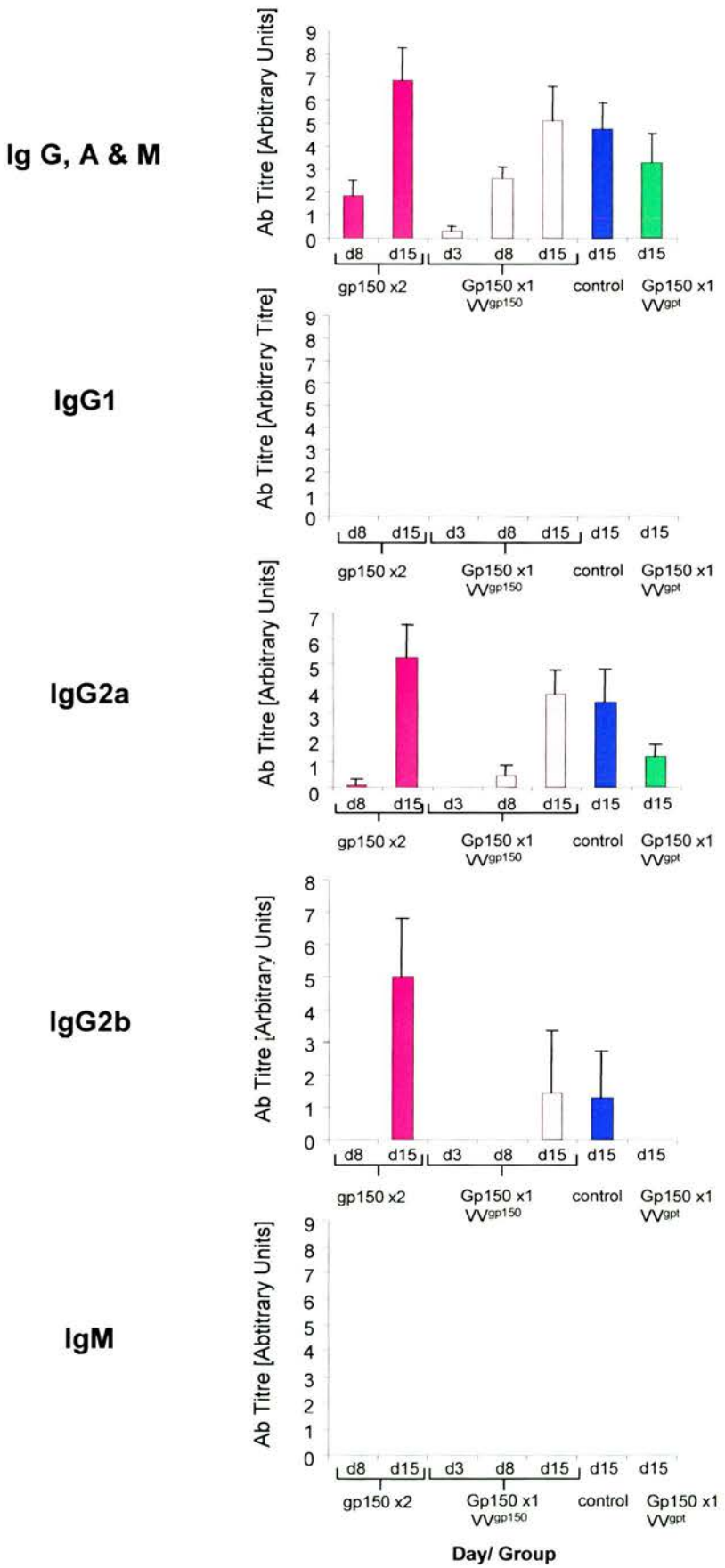


Figure 3.6.8. Analysis of immunoglobulin subclass of the MHV-68 specific antibody response post MHV-68 challenge in C57BL/6 mice vaccinated with different regimens. Sera was prepared from blood taken from C57BL/6 mice that had been vaccinated with different gp150 based regimens pre and post infection with MHV-68 (days 3, 8, 15, 29 and 59). An enzyme linked immunorbent assay was used to determine the levels of different immunoglobulin subclasses of MHV-68 specific antibody in samples relative to a standard curve generated using dilutions of mouse anti-MHV-68 hyperimmune antiserum. Vaccine regimens included priming animals with plasmid pVR1255/gp150 (gp150 DNA) followed by a booster three weeks later of either gp150 DNA, or one of two recombinant vaccinia viruses - VV^{gp150} and VV^{gpt}. VV^{gp150} encodes gp150 and the *E.coli* guanosine phosphoribosyl transferase gene (*gpt*), the control, VV^{gpt}, contains *gpt* alone. Plasmid was administered using gene gun delivery to the abdomen of mice, one shot (1μg DNA) per plasmid. Intranasal administration of 2×10^6 pfu recombinant vaccinia virus was done. The unvaccinated control group received MHV-68 alone. Data represent mean values from four mice, bars indicate standard deviation. The data points positive for anti-MHV-68 specific antibody within the red dotted lines in figure 3.6.7 were selected for analysis of immunoglobulin isotype populations.

4.1.1 DISCUSSION

Gp150 is a potential virus to cell attachment facilitating protein for MHV-68 due to its virion surface localisation, induction of neutralising antibodies and similarities to gp340/220 of EBV. In order to investigate the function of gp150 two main approaches were taken. The generation of a gp150 negative recombinant virus and functional analysis of isolated gp150 in binding studies. Furthermore, a preliminary investigation into the practical application of gp150 as a DNA vaccine combined with a recombinant vaccinia virus expressing gp150 has been performed to determine if protection against MHV-68 infection can be achieved.

4.1.2 Generation of a Gp150 Negative Recombinant Virus

Generation of recombinant herpesviruses dysfunctional in a specific gene by homologous recombination in mammalian cells has been used successfully to determine the function of a broad range of herpesvirus genes including glycoproteins. Here, the green fluorescent protein (GFP) encoding gene was targeted for homologous recombination into the *gp150* locus of the MHV-68 genome. This would result in a recombinant virus unable to express gp150 and identifiable by the expression of GFP. The first plasmid construct used, when co-transfected with MHV-68 DNA, successfully resulted in the generation of recombinant viruses expressing GFP. However, DNA analysis of virus cultures which appeared pure following sequential plaque picking i.e. wild type plaques were absent, indicated that the entire plasmid had recombined into the MHV-68 genome. Southern blot analysis indicated that the plasmid had integrated into the genome directly to the left of the gp150 gene via a single crossover event. This was similar to the experience of Lee *et al.*, in their generation of EBV recombinant viruses (Lee *et al.*, 1992). Although not useful in terms of a gp150 knock out virus this was promising from the point of view that the single recombination event appeared specific, targeting *gp150.*

If gp150 is essential for virus replication a virus unable to express gp150 would be at a growth disadvantage and therefore, during plaque picking and limiting dilution used to purify recombinant virus this would be selected against. To adapt to this possibility a second recombinant virus vector construct for homologous

recombination was generated specifically to be compatible for recombinant virus growth in a gp150 complementary cell line. The level of sequence in the recombinant construct homologous to gp150 sequence carried by the cell line was minimised. Recombinant viruses grown in complementary cell lines have been known to revert to wild type following recombination with the intact gene carried by the cell line, as was experienced by Prichard *et al.*,1999, when producing a HCMV recombinant negative for an essential gene (UL84). Cultivation of recombinant viruses on a cell line expressing gp150 should enhance the selection of gp150 negative viruses. Recombinant viruses expressing GFP were successfully generated using this new construct, sequential plaque picking and limiting dilutions of virus on normal cells resulted in enrichment of green virus however wild type virus persisted. Southern blot analysis of DNA from virus cultures indicated that the recombinant gene cassette containing *gfp* had not integrated into the virus genome at the targeted site. The data shown here suggests the recombinant cassette had inserted to the right of the centre of the genome in the region of ORF58. The function of ORF58 is not known. The adjacent ORF57 is an immediate early gene, although not directly characterised it is likely to encode a transcriptional activator considering the known function of the EBV and HVS ORF57 homologues (Virgin *et al.*, 1997). Why the recombinant construct has inserted here is open to speculation. This area may be transcriptionally active during lytic viral replication and so accessible for recombination. The generation of a complementary cell line expressing gp150, to aid isolation of a gp150 knock out virus, was unsuccessful and therefore was not available for use in recombinant virus purification.

4.1.3 Modifications for the Generation of Recombinant Viruses

From the process used here to generate recombinant viruses several aspects require attention for improvement. Enhancement of the probability of targeted recombination occurring may be achieved by increasing the size of flanking sequences. The production of a new recombinant construct with flanking sequences for homologous recombination each of approximately 5 kilobase pairs was underway towards the end of the project but not completed. Equally important to homologous recombination is the efficiency of recombinant virus purification and analysis. In

order to accurately characterise the phenotype resulting from the absence of a particular gene product the virus must be clonal and free from wild type virus contamination. GFP serves as an excellent marker tool to identify the presence of recombinant viruses. However, using physical isolation of recombinant virus by sequential plaque picks and limiting dilutions was not fully successful at eliminating the presence of wild type virus. Unstable insertion of the recombinant cassette resulting in loss of the cassette from the genome could also cause the presence of wild type virus to be detected. In this regard the use of a drug resistance encoding gene for positive selection of recombinant viruses could prove very valuable. In fact, initial work was performed to construct a vector for homologous recombination containing both *gfp* and the gene encoding the *E. coli* guanosine phosphoribosyl transferase gene (*gpt*) flanked by the genomic targeting sequences. *Gpt* has been used successfully to select Marek's disease virus and HCMV recombinants (Marshall *et al.*, 1993, Greaves *et al.*, 1995). Unfortunately incorporation of both genes into vector pSP72/gp150 proved incompatible. Introduction of the hygromycin phosphotransferase gene would allow positive selection of hygromycin resistant viruses (Wang *et al.*, 1991, Marchini *et al.*, 1992). Other selectable markers that have been used for selection of herpesvirus recombinants include the HSV-1 thymidine kinase gene (Post *et al.*, 1981) and the neomycin resistance gene (Neidhardt *et al.*, 1987, Wang *et al.*, 1998). In summary large flanking sequences (in the kilobase range) and use of a positive selection marker such as the hygromycin resistance gene are strong recommendations for the generation of recombinant viruses in the manner used here.

The isolation of a recombinant virus resulting from the desired homologous recombination crossover event may have been hindered by the process of plaque purification selecting against viruses with debilitated growth. More rapid identification of the required recombinant is needed that would eliminate time consumed purifying incorrect recombinants prior to analysis of their DNA. A PCR assay was designed in this vein in order to identify from the initial transfection whether the required recombination event had occurred using a primer from within the GFP gene and one located in the MHV-68 genome, out with the region of

recombination. Unfortunately, in analysis of transfections using the second recombination construct no product was detected suggesting the required recombination had not occurred. This detection method does have potential, detection of the desired recombination in the total DNA extracted from an initial transfection would allow one to pursue isolation of the sought after recombinant with confidence. Many clones could be screened early on by PCR rather than growing clones up for southern blot analysis. Ultimately, isolation of the correct recombinant is a numbers 'game' i.e. the more virus clones analysed the more chance of identifying the correct recombinant. Better targeting of recombination using larger flanking sequences and the use of a marker for selection should improve the probability of success.

Infection of B cell lines with recombinant virus mixes and cloning the cells could be a more effective method at isolating recombinant viruses. An advantage of using B cells is that lytic replication of the virus is not essential i.e a virus genome negative for an essential lytic gene can be propagated as a latent episome in dividing B cells. The transfection efficiency of B cells is low in comparison to adherent cell lines (a well known phenomenon) therefore the initial transfection may have to be performed in adherent cells lines with subsequent infection of B cells. In addition the detectable marker GFP, under the control of the CMV promoter may be subject to shut down in B cells due to the establishment of the latent form of the viral genome. Incorporation of a selectable marker such as hygromycin resistance into recombinants would allow selection of recombinants in this system. *In vitro* the majority of MHV-68 infected cells in a B cell line are latently infected however there is a low level, approximately 5%, of lytic virus replcation (Sunil Chandra *et al.*, 1993). Chemical agents such as the phorbol esters e.g. 12-O-tetradecanoyl-phorbol-13-acetate can be used to activate lytic replication and therefore produce progeny virions from the recombinant virus genome. EBV recombinants engineered with resistance to selective agents have been successfully isolated by cloning in B cells (Marchini *et al.*, 1992, Wang & Hutt-Fletcher, 1998).

4.1.4 Alternative Strategies for Generating Recombinant Viruses

A major drawback of the method used here for the generation of recombinant viruses was the contamination by wild type virus. The use of cosmids is an alternative method to generate recombinant herpesviruses in the absence of wild type virus (Cunningham & Davison, 1993, van Zijl *et al.*, 1988, de Wind *et al.*, 1990). For this a cosmid library of the viral genome is used which usually consists of approximately 5 cosmids that each contain large stretches of DNA, in the region of 30 – 40 kb, from the viral genome. The gene of interest can be modified i.e. mutated in the particular cosmid it is located within and cloned in bacteria. The genomic insert of this cosmid is excised and in conjunction with excised inserts from cosmids containing sections of the rest of the viral genome in overlapping stretches (overlapping from 5 to 20 kb) is transfected into mammalian cells. Virus is produced following recombination of the lengths of genomic DNA at homologous sequences resulting in the generation of an intact virus genome. The gene of interest must also be a unique sequence in the cosmid library and not in a stretch repeated in several cosmids. This method of recombinant virus generation requires a cosmid library of the virus genome which takes time to generate however a comprehensive library could be extremely valuable in the long-term. Another advantage with the cosmid system is that a marker is not required and therefore discrete mutations of the target gene i.e. a few base pairs can be done rather than inserting additional DNA which may be more disruptive. Correct recombination of the fragments needs to be verified by Southern blot analysis. Most systems have their inherent difficulties, one of which is the instability and subsequent loss of repeat sequences, common to herpesvirus genomes, as they are cloned in bacteria (Gray & Kaerner, 1984, Quinn & McGeoch 1985, Weller *et al.*, 1985). In addition the restriction site used to excise the DNA inserts from the cosmids must not be present in the viral genome.

The use of the bacterial artificial chromosome (BAC) system to generate recombinant viruses is another alternative method which avoids the problem of contamination of recombinant virus with wild type virus. In the BAC system the entire intact viral genome is manipulated in bacteria in the absence of viral gene expression and generation of virions. To engineer the viral genome into a bacterial

artificial chromosome the genome is cloned onto a F (fertility) factor based plasmid which can encompass large foreign DNA fragments (up to 300kbp) (Shizuya *et al.*, 1992). This is done by conventional homologous recombination of the F factor plasmid flanked by viral genomic sequences with viral DNA following their co-transfection into eukaryotic cells. Recombinant viral DNA containing the F factor is purified from these cells and introduced into *E. coli* by electroporation. Genetic manipulation of the viral genome can be done in bacteria The structure of the manipulated genome i.e. successful elimination of a particular gene and insertion of marker genes if desired can be analysed and confirmed prior to introduction of clonal recombinant virus DNA into mammalian cells for the production of infectious progeny. In this way virus produced is of known genotype. This is a major advantage of the BAC system in comparison to using cosmids where the correct recombination of the genomic fragments needs to be verified. Furthermore, isolated viral genome DNA with essential genes made dysfunctional can be achieved and cloned in bulk in *E. coli* without the need for a complementary cell line. That is to say, construction and isolation of the recombinant genome is independent of the biological fitness of the mutant virus.

The use of the BAC system for production of herpesvirus recombinants is increasing, viral genomes which have been incorporated into BACs include MCMV, HSV and EBV (Delecluse *et al.*, 1998, Messerle *et al.*, 1997, Horsburgh *et al.*, 1999). The as yet limited use of the BAC system stems from the time taken to generate a BAC genome and the unfamiliarity of this new technology. The greater stability of HSV sequences in BACS in comparison to HSV sequences in cosmids is attributed to the low copy number of BACs per cell (i.e. one copy). The availability of a BAC incorporating the MHV-68 is the restricting factor at present for the use of the BAC system. In this laboratory, work is underway to produce such a tool which once established may be adopted for the efficient generation of MHV-68 recombinants in future. Koszinowski *et al.* report they have a MHV-68 BAC (Adler *et al.*, 1999). Successful use of the BAC system would eliminate the lengthy rounds of selection and analysis of recombinant clones inherent with conventional recombinant techniques. It remains to be seen if the BAC system will live up to the promise and

prove to be the solution for efficient generation of MHV-68 recombinant viruses. The stability of the genome in bacteria needs to be monitored.

4.1.5 Mammalian Expression of Gp150 – pBabe/PgBgp150

A plasmid construct, pBabe/PgBgp150, was made consisting of the gene encoding gp150 positioned downstream of the putative gB promoter (PgB) to be used for the generation of a complementary cell line inducible for the expression of gp150 following virus infection. The intention was to use this cell line in conjunction with the generation of a gp150 negative recombinant virus. In the event that gp150 is essential for virus replication the cell line would allow culture of gp150 negative viruses. Inducible expression of gp150 was chosen to avoid possible problems of toxicity known to occur following constitutive expression of some viral glycoproteins. Although a C127 cell line containing pBabe/PgBgp150 was selected using puromycin and the presence of gp150 DNA confirmed by PCR the expression of gp150 was not detected. In the absence of viral infection low level expression of gp150 could have occurred via the strong promoter in the long terminal repeat derived from the murine Moloney leukemia retrovirus upon which the plasmid backbone was based (Morgenstern & Land, 1990). Following infection of the cell line with MHV-68 the levels of gp150 mRNA and protein did not differ in comparison to control cells. Higher levels of gp150 in the complementary cell line following infection with MHV-68 DNA compared to MHV-68 infected control cells would have indicated that gp150 expression via the gene encoded by the cell line had occurred. In the absence of this it appeared that the cell line generated did not express gp150.

The design of the pBabe/PgBgp150 plasmid was based on knowledge of a successful complementary cell line, in which the HSV-1 gH gene was under the control of the gD promoter, generated for propagation of a gH negative recombinant HSV-1 (Forrester *et al.*, 1992). Following infection of these cells with HSV a much greater level, at least 10 fold, of gH was detected by western analysis, in comparison to normal cells infected with HSV-1. With the availability of the closely related HSV-2 they were able to induce expression of gH by infection of the complementary cell

line with HSV-2 and distinguish the expression of the cellular encoded HSV-1 gH from the viral HSV-2 gH using monoclonal antibodies specific to gH of HSV-1. Unfortunately a similar method for testing the expression of gp150 from the cell line – other than assessment of increased level of gp150, was not available. Other gammaherpesviruses such as acelaphine herpesvirus 1 or KSHV may be closely related enough to transactivate MHV-68 promoters, however this has not been tested and the availability of other gammaherpesviruses such as a AHV 1 is limited by their restricted growth *in vitro*. The putative gB promoter consisted of a 264bp region directly upstream from the translational start site of the gB gene (Stewart *et al.*, 1994), although not experimentally confirmed as the promoter it was likely that this region was the gB promoter. A TATA box is present 67bp upstream from gB and viral promoters in herpesvirus genomes are commonly located directly before genes for glycoprotein expression. In the case of the HSV-1 gD promoter all the signals necessary for full regulated expression lie within 83bp of the RNA start site and some promoter activation is still detectable when only 55bp upstream are present (Everett, 1983). Uncertainty does however remain as to whether this putative PgB promoter is functional. Insertion of a reporter gene such as *gfp* downstream of PgB could be used to determine if expression via this promoter occurs upon infection of a cell line containing this construct. An alternative approach would be to design a primer from the transcritional start site of the putative gB promoter and pair this with a primer from within gp150 in an RT-PCR to detect gp150 mRNA transcripts derived from the vector. However, analysis of RNA expression following infection with MHV-68 showed the presence of one transcript, if expression from the cell line occurred another gp150 positive transcript of at least a slightly different size might be expected to be present. A tag engineered to the C terminus of gp150 such as a hexahistine tag would allow identification of cellular encoded gp150 to be distinguished from that of viral gp150.

Possible reasons for lack of gp150 expression from pBabe/PgBgp150 could include mutation of the plasmid backbone or integration of the vector into the host cell genome into a site inaccessible for expression. The PgBgp150 insert was sequenced and shown to be intact. Of the clones generated that were positive for gp150 DNA

the clone that provided the greatest signal by PCR for the presence of gp150 DNA was selected for further analysis of gp150 expression (i.e. Northern blotting and radioimmunoprecipitation). By immunofluorescence and western blotting all clones were negative for gp150 expression. Further analysis of other clones might prove fruitful. In the absence of a gp150 negative recombinant virus and the uncertainty as to whether a complementary cell line would be essential this system was not pursued further. Of course, a functionally confirmed complementary cell line may be used successfully to isolate gp150 negative viruses that may have debilitated growth on normal cells. That is to say, existence of a gp150 negative virus might come after the generation of a complementary cell line required as a tool to isolate gp150 null mutants.

4.1.6 Ecdysone Inducible System for Mammalian Expression of Gp150

In order to express gp150 in mammalian cells an alternative system was adopted in the form of the ecdysone inducible system. The advantage of this system is that expression of the inserted gene i.e. gp150 can be controlled externally by the addition of the induction agent Ponasterone A (an analogue of ecdysone). This therefore avoids constitutive expression of gp150 which could prove toxic, a feature of glycoproteins that has hindered generation of cell lines for their expression (Whang *et al.*, 1987, Conway *et al.*, 1989, Motz *et al.*, 1987, Gompels & Minson, 1989) and in addition induction of gp150 is independent of viral infection. Not only could such a cell line be used to complement a gp150 negative virus but comparison of the susceptibility of the same cell line to MHV-68 infection in the absence and presence of gp150 expression may provide indications as to the role of gp150. For example reduced ability of MHV-68 to bind to or enter cells expressing gp150 may indicate that the cellular expressed gp150 interacts with and sequesters cell surface molecules which virion gp150 requires to interact with for infection to occur. This scenario has been shown for gD of HSV-1 (Campadelli-Fiume *et al.*, 1990, Geraghty *et al.*, 2000). A construct was also engineered containing the region encoding the extracellular domain of gp150 in order to produce soluble gp150 which should be secreted into the culture media in the absence of the hydrophobic transmembrane anchoring domain.

This was intended as a source of soluble gp150 to be used in functional studies such as determining the proposed binding function of gp150.

Expression of β-galactosidase from a control plasmid using the ecdysone system was achieved confirming that the plasmid peparations and the induction agent were functional. However, gp150 expression was not detected from any of the three constructs generated. Use of different cell types and levels of induction agent did not have any detectable effect. Selection of cells containing both the expression and ecdycone receptor encoding vectors was done to enhance the number of potential cells expressing gp150 over that of a transient transfection. No gp150 expression was detected. The sequences of the gp150 inserts were determined to be correct therefore mutation of the insert was ruled out as the cause of lack of detectable expression. The inserted sequences included their own ATG and Kozak translation initiation sequence. The expression plasmid backbone may have undergone mutation inhibiting expression of the insert. Perhaps something inherent within the gp150 gene is incompatible for expression from this vector. Although a ready made control expression vector is provided, encoding β-galactosidase, it would be worth cloning a reporter gene, and other genes in general for that matter, into the expression vector used to determine if expression of genes other than gp150 is successful. Analysis of RNA expression would provide an indication of whether gp150 encoding mRNA is generated and so suggest if lack of detectable protein expression was due to the lack of transcription or efficient translation. The expression vector provides the SV40 pA downstream of the gp150 insert which is designed to increase stability of the mRNA transcript. Although glycoproteins can be toxic to cells, short term expression of gp150 during transient transfection i.e 24- 48 post transfection is unlikely to be long enough to cause cell decline and lack of detection gp150. The reason for lack of expression of gp150 using the ecdysone system is unclear.

4.1.7 PcDNA3 & PcDNA3.1 for Expression of Gp150

The gene encoding gp150 was cloned into pcDNA3, downstream of the constitutive CMV immediate early promoter, for mammalian expression of gp150 with the aim of using this vector as a DNA vaccine to induce immunity against MHV-68. In

addition the vector could be used to generate a cell line constitutively expressing gp150. The CMV immediate early promoter is one of the strongest enhancer/promoters known (Boshart *et al.*, 1985, Thomsen *et al.*, 1984, Ghazal *et al.*, 1987). Expression of gp150 from pcDNA3/gp150 and pcDNA3.1/gp150 was not detected. PcDNA3.1 is an updated version of pcDNA3 with a more extensive multiple cloning site (MCS) and lacks the bacteriophage SP6 promoter site present in pcDNA3 positioned just downstream of the MCS into which genes for expression are inserted. The production of an updated version of pcDNA3 is perhaps a testament to the inefficient expression of this vector. The manufacturers indicated that the SP6 promoter site has been removed as it is thought to cause secondary structures in RNA transcripts that are detrimental to protein expression (personal communication, Invitrogen) presumably due to inefficient association of transcripts with ribosomes. From discussions with other workers within and out with this laboratory the failure of pcDNA3 to yield expression of the inserted gene is not an isolated event. It is possible that there is low level expression of gp150 below the level of detection. In a pcDNA3 construct containing the M2 ORF of MHV-68 no expression was detectable by conventional immunofluorescence and western blot analysis however it appears that a cell line containing this vector could act as a target for M2 specific cytotoxic T cells (A McCrae & E Usherwood, personal communication) suggesting that a very low level of M2 protein expression was present, enough to result in processing and presentation of M2 via the MHC I pathway. None the less, with regards to vaccination it would be preferable to use a vector that provides efficient expression of gp150 and leaves no doubt over the generation of the protein. Such a vector exists in the form of pVR1255/gp150.

4.1.8 PVR1255/gp150 Expression of Gp150

It must be stressed that the vector pVR1255/gp150 was acquired at a late stage in the project. Confirmation of expression of gp150 in eukaryotic cells from this vector was achieved rapidly by conventional immunofluorescence and western blot analysis of transfected cells. This vector was deemed suitable for use as a gp150 DNA vaccine. The successful expression of gp150 using vector pVR1255 begs the question as to how this construct differs from the other vectors engineered here for

the expression of gp150. This vector confirms that it is possible to express gp150, at least transiently, from a mammalian expression vector. Within pVR1255/gp150 the entire gp150 encoding region, from the translational start codon to the stop codon, is inserted downstream of the constitutive CMV promoter. Of note is the presence of the CMV immediate early intron A sequence positioned between the CMV promoter and the gp150 gene (for a map of the plasmid refer to figure 3.3.2). The intron A sequence contains binding motifs for transcription factors such as nuclear factor 1 and is known to enhance expression of inserted genes (Chapman *et al.*, 1991, Hartikka *et al.*, 1996). The intron A element is not present in pcDNA3 and pcDN3.1. The pcDNA3 vectors differ from pVR1255 in that they have a bovine growth hormone polyadenylation signal (BGHpA) positioned downstream of the inserted gene whereas pVR1255 has a minimal transcriptional terminator derived from the rabbit β globin gene (mRBGpA) (Levitt *et al.*, 1989). The expression of a luciferase insert from pVR1255 was reported to be two fold greater when compared to an earlier version of the vector which differs only in that it contains a BGHpA as opposed to the mRBGpA (Hartikka *et al.*, 1996). In a range of vectors studied by Hartikka *et al.*, to determine plasmid elements required for optimum gene expression they unexpectedly found replacement of the ampicillin gene with the kanamycin resistance gene resulted in an over two fold increase in expression of luciferase (Hartikka *et al.*, 1996). Whether this effect depends upon the context of the resistance gene or if specific features of the kanamycin gene result in better expression of the inserted gene in expression vectors in general (i.e. not just the ones tested by Hartikka *et al.*) in comparison to vectors encoding ampicillin resistance is not known. It is worth noting however that pcDNA3 encodes ampicillin resistance in contrast to the kanamycin resistance carried by pVR1255. Another difference in the plasmid backbone is that the pcDNA3 vectors used here encode the neomycin resistance gene whereas pVR1255 does not encode any marker for drug selection in eukaryotic cells.

The work of Hartikka *et al.* 1996, systematically adding or replacing elements of plasmid expression vectors, demonstrates that introduction of new DNA sequences can have dramatic and unexpected effects on expression of the target gene. There

may be unidentified features of the gp150 gene that make it incompatible for expression in certain vectors such as pcDNA3.

As well as use as a DNA vaccine pVR1255/gp150 offers much promise particularly in the light of failure to express gp150 using other vectors. PVR1255/gp150 could be used to generate a mammalian cell line expressing gp150 for complementation of a gp150 negaitve virus. Deletion of the hydrophobic transmembrane encoding domain of gp150 within the vector would in theory result in secretion of gp150 from cells transfected of the modified pVR1255/gp150 plasmid. This could be used as a source of eukaryotic derived gp150 for binding studies. One disadvantage with pVR1255/gp150 in terms of generation of a mammalian cell line expressing gp150 is that the vector lacks a eukaryotic selectable marker. Modification of the vector by insertion of a eukaryotic selectable marker is feasible but of course alterations in the vectors backbone could have adverse effects on protein expression. Alternatively co-transfection of pVR1255/gp150 with a vector containing a eukaryotic selectable marker and selection of double transfectants could be done. Due to the efficient expression displayed by this vector many workers in the laboratory have adopted pVR1255 for expression of a range of viral genes.

There are no definitive reasons why several different plasmid constructs failed to express gp150 in mammalian cells. Speculation includes any one of a number of general reasons why protein expression is not detected. Sequence analysis has ruled out the lack of expression due to corruption of the insert. Unstable or structurally deformed mRNA, insertion of the plasmid into areas of the host cell genome that are inaccessible for transcription may result in shut off of gene expression. Although the inserts for all expression constructs generated were shown to be intact by sequence analysis it is possible that the vector backbone had mutated. However, this seems unlikely to be the reason for lack of expression for all the different vectors used. To establish this it would be necessary to sequence each entire plasmid backbone (>5kbp) which would be expensive in both time and money.

The gp150 gene may be incompatible for expression in certain vector contexts. An interesting feature to note is that growth of *E. coli* transformed with pVR1255/gp150 was restricted in comparison to growth of *E. coli* transformed with pVR1255 containing other inserts such as the interferon alpha type 1 encoding gene (personal communication, Elenora Arico). In addition, low yields (approximately 20 fold) of pSP72/gp150 DNA in comparison to that of pSP72 were produced following large plasmid preparations. The colonies formed by bacteria transformed with pcDNA3/gp150 were noticeably smaller than those produced from bacteria transformed with pcDNA3 alone. Furthermore, in the generation of a cosmid library of the MHV-68 genome the isolation of a cosmid clone containing a ~2kb stretch of the genome which includes the gp150 ORF is proving difficult. The rest of the genome has been isolated in cosmid clones apart from this elusive stretch (A. Davison, personal communication). These observations suggest some property of gp150 is not highly compatible with standard cloning. This may be due to the high AT content or the proline rich repeat sequences of the gp150 encoding gene.

4.1.9 Binding Analysis of Gp150

For the source of isolated gp150 for functional studies bacterial expression and purification of the extracellular portion of gp150 as a fusion protein attached to a hexahistidine tag was achieved. The gp150-His fusion protein was sufficiently similar to eukaryotic gp150 for identification by rabbit MHV-68 specific antiserum. In addition a GST-His fusion protein was generated as a control protein for functional studies of gp150-His.

The ability of gp150-His to bind to different cell types was analysed in order to determine if gp150 is involved in virion attachment and entry of target cells. Significant binding of gp150-His (at 20μM) to approximately 3% of 10^6 splenic lymphocytes was observed. The shape of the FACS graphs showed the whole lymphocyte population shifted in fluorescence following incubation with gp150-His suggesting gp150-His bound the heterogeneous lymphocyte population as a whole. As opposed to the appearance of a second peak with greater fluorescence intensity

which would suggest a particular subset of cells present with a high affinity for gp150-His. In further analysis gp150-His exhibited binding to purified splenic B cells, approximately 6% of cells were bound. Double staining of splenic lymphocytes to determine the CD19 phenotype of bound cells showed gp150-His bound to approximately equal numbers of $CD19^+$ and $CD19^-$ cells in a heterogeneous population of splenic lymphocytes. Indicating that gp150-His binds both B lymphocytes and non B lymphocytes. In contrast no significant binding of gp150-His to C127 murine epithelial cells or inhibition of MHV-68 infection by gp150-His was detected. These observations suggest that gp150-His binds a lymphocyte cell surface marker absent from or in insufficient levels in C127 epithelial cells.

From this data it is not possible to rule out gp150-His binding to epithelial cells in general as lack of binding may be due to a feature specific to C127 epithelial cells. Airway epithelial cells which MHV-68 encounters following intranasal infection of mice may differ in their binding potential for gp150-His. Binding to primary splenic lymphocytes is shown. It would be advisable to test a wider variety of epithelial cells to determine if lack of binding to C127 cells is a feature specific to this particular cell line or if gp150-His is unable to bind to adherent cells in general. Gp150-His may bind a lymphocyte specific marker however, the testing of further epithelial cell lines is required to establish a clearer picture.

The number of splenic lymphocytes bound by gp150-His appears low considering the relatively high concentration of protein applied (20μM) which is equivalent to 5 x 10^8 molecules per cell. The level of protein used here is towards the high end, of protein concentrations used in similar binding studies (Hedrick *et al.*, 1994, Johnson *et al.*, 1990, Tanner *et al.*, 1987). This may suggest that there is a low percentage of cells able to bind gp150-His in the splenic lymphocyte population. The affinity of the interaction is not known. It may be that the cells bound express a high level of the gp150-His binding molecule/molecules, the affinity may be low and so cells expressing lower levels of the required molecules may not exhibit significant binding of gp150-His.

Using the FACS assay MHV-68 was shown to bind to approximately 10% of splenic lymphocytes when 10^7 pfu was added to 10^6 splenocytes. Double staining FACS data shows MHV-68 binds both $CD19^+$ and $CD19^-$ lymphocytes. Of total lymphocytes bound by MHV-68 in the assay reported here, only 20% were $CD19^+$. This could be considered surprising for a B cell tropic virus where 80% of the cells bound from the heterogeneous lymphocyte population are not B lymphocytes. However, attachment to cells does not necessarily equate to infection of the cell and tropism of MHV-68 may not be determined by the receptor MHV-68 uses for attachment. In addition, MHV-68 can infect a wide range of cells including epithelial, fibroblastoid, neuronal and T cells which suggest MHV-68 may use a rather more ubiquitous cellular receptor in comparison to EBV. Dutia *et al.*, detected non B lymphocytes that were readily susceptible to MHV-68 infection (Dutia *et al.*, 1999a).

Using FACS analysis approximately 60% of the C127 epithelial cells were bound by MHV-68 when 10^7 pfu was incubated with 10^6 cells. This represents a much greater proportion of the population of the cells bound by MHV-68 in comparison to the proportion of cells bound in the hetereogeneous splenic lymphocyte population or purified B cells. This may be due to more receptors present on C127 epithelial cells and the greater surface area of epithelial cells increasing probability that virion comes into contact with cells. The affinity of interactions of MHV-68 with splenocytes in comparison to epithelial cells has not been determined. A quantitative study of the cell types bound by MHV-68 and the level of MHV-68 binding could be valuable in providing a key as to what MHV-68 specifically binds to on the surface of cells. As yet a cell type which is not susceptible to infection by MHV-68 has not been identified – many different cell lines have been tested but the list is by no means comprehensive and does not include cell lines with specific defects such as heparan sulphate negative murine L cells (Gruenheid *et al.*, 1993).

The FACS analysis used here allows the proportion of cells bound by gp150-His or virus to be determined however, the proportion of gp150-His or virus bound from the total added is unknown. That is to say, the FACS assay is not quantitative and so information such as the number gp150-His molecules or virions bound or the affinity of binding is not determined. In order to determine such information fusion protein molecules and virions need to be directly labelled to allow quantitative detection of levels bound. Radiolabelling fusion protein molecules/virions with isotopes such as ^{125}I can be done and the level of radioactivity associated with cells following incubation with different concentrations of the fusion protein molecules or virions measured. Quantitative analysis of binding would allow the protein and virion concentration causing saturation to be determined and used to calculate the number of receptor sites per cell for the protein or virus. Radioiodination can have adverse affects on protein or virus for example Hedrick *et al.*,1994, found tyrosine iodination compromised binding ability of recombinant gp350/220.

The particle to pfu ratio of the virus preparations used was not known. At 10^7 pfu of virus there may be a log more virions capable of binding depending on the particle: pfu ratio. Therefore caution must be used to avoid making assumptions about the level of virus bound in the absence of quantitative data.

Although use of radiolabelled protein and virions would provide a quantitative handle on the level protein or virus of binding the FACS analysis used here enables the number and type of cells bound to be determined. Further use of double staining for other cell surface markers besides CD19 might provide information on the type of cells within the heterogeneous lymphocyte population bound by gp150-His and MHV-68. MHV-68 may favour attachment to and infection of cells at a particular stage in development which can be detected by surface markers that are present during specific stages of B and T lymphocyte maturation. The virus receptor may be a common molecule that needs to be present at a certain level to enable efficient attachment of the virus.

Analysis of the effect of incubation of gp150-His with splenic lymphocytes may indirectly indicate the receptor to which it binds. That is to say, binding of cells by gp150-His may result in changes in the cell such as activation and/or proliferation if it binds to a cell surface molecule involved in signal transduction. Gp340/220 binding to CD21 of B lymphocytes pushes resting B cells into the cell cycle (Sinclair *et al.*, 1994). Analysis of levels of cell surface markers upregulated on activated lymphocytes and the level of proliferation of cells could be tested in the presence and absence of gp150-His.

Whether gp150-His is binding to the same site on splenic lymphocytes as virus has not been determined. The ability of gp150-His to block virus binding to lymphocytes would provide an insight into this. If virus binding was blocked by gp150-His it would suggest that gp150-His binds the same receptor as MHV-68. In the absence of blocking there are a number of scenarios that could be correct. For example if gp150-His binds to the same receptor but with a lower affinity the virus expressed gp150 may dislodge the gp150-His. The concentration of the protein is too weak and/or the binding affinity too low. Even if one molecule of gp150-His and one molecule of gp150 at the virion surface have the same affinity for a receptor due to the multiple number of molecules at the virion surface for interaction with the cell surface the virion may have a higher binding avidity in comparison to a single molecule of gp150-His. Blocking of virus binding to splenocytes by gp150-His was not tested using the FACS analysis system because an antibody that would distinguish MHV-68 binding from that of gp150-His was not available. Further work could include testing if virus can block gp150-His binding to cells by using the anti-His monoclonal antibody.

Binding of gp150-His to splenocytes has been shown indicating that gp150 can bind to lymphocyte surface molecules. Demonstration of binding alone does not necessarily mean that a protein is required for the initial attachment of the virus to cells, the protein may have a post binding entry role. This is the case for gD which is involved in cell penetration by HSV-1, gD binds to the cell surface but is not essential for virion attachment to cells (Ligas & Johnson, 1988, Johnson *et al.*, 1990).

That is to say, although gp150-His has been shown to bind to cells the exact function of this binding property for gp150 could be for the proposed attachment of MHV-68 to cells but alternatively it could have a post attachment entry role.

In order to substantiate the observed lymphocyte binding ability of gp150-His identification of the molecule bound by gp150-His is strongly recommended. To identify the receptor gp150-His is binding to a range of approaches could be taken. Analysis of common features of the cells bound by gp150-His could provide an insight e.g. pan-lymphocyte specific markers. A more direct and recommended approach is that of 'receptor pull down' analysis using cross-linking agents to covalently link gp150-His to the cellular molecule/molecules it interacts with and subsequently immunoprecipitate gp150-His plus the attached cellular receptor using gp150 specific antiserum. Indeed, a preliminary assay using ^{125}I labelled splenocytes was performed in this manner however, due to the number of parameters involved limitations of time inhibited establishment of the assay and full analysis by this method.

Another method that could allow identification into what gp150-His binds to on the cell surface is the use of surface plasmon resonance (SPR). SPR allows the detection of very subtle interactions between molecules. Gp150-His could be coupled to the surface of a SPR biosensor chip. Solubilised lymphocyte membrane preparations passed over the immobilised gp150-His protein may allow the cell surface molecule or molecules which gp150 interacts with to associate with gp150. Molecular interactions with the immobilised protein result in changes in the surface of the chip and changes in diffraction of light directed to the underside of the chip are measured. Measurements are taken in real time and so allow on/off rates and the affinity of protein interactions to be calculated. The captured protein which has bound to immobilised protein can be eluted and then characterised. SPR is proving an extremely powerful tool to identify and analyse molecular interactions. Influenza haemagglutinin binding to sialic acid receptors (Takemoto *et al.*, 1996), soluble intercellular cell adhesion molecule binding to human rhinovirus (Casasnovas *et al.*, 1995) and HSV gB binding to glycosaminoglycans (Williams & Straus, 1997) have

been analysed using SPR. The binding ability of truncated forms of gD to the herpesvirus entry mediator has been analysed in detail using SPR also (Willis *et al.*, 1998). If the molecule to which gp150-His binds is identified experiments to test the importance of this molecule in the context of the virus lifecycle could be done. For example antibodies against the molecule could be tested to see if they inhibit virus binding and/or infection of cell.

A virus overlay protein assay (VOPBA) failed to detect a cellular protein to which MHV-68 virions or gp150-His bound. As described in section 3.2.7 VOPBAs are only useful in certain circumstances where binding is not dependent on protein conformation or affected by denaturation, a negative result by VOPBA alone by no means rules out lack of binding function of a virus or protein (Boyle *et al.*, 1987). As mentioned a cell line which cannot be infected or bound by MHV-68 has not been identified therefore experiments in which a wide range of different molecules are each expressed in cells from a mouse cDNA library in an attempt to identify one which facilitates virus binding are not feasible as yet.

From here the use of gp150-His in quantitative cell binding studies and identification of the cellular molecule to which gp150 interacts is suggested. The bacterial expression of gp150 has been advantageous in ease of expression and purification and has provided valuable indications on the ability of gp150 to bind to splenic lymphocytes. It must be remembered that differences in gp150-His due to the expression in a heterologous host may cause the function of gp150 to be altered or diminished compared to gp150 expressed in eukaryotic cells. It is recognised that caution must therefore be taken when interpreting the function of virion gp150 from data that has been generated using a protein that has been produced artificially. Lack of significant binding of gp150-His to C127 cells does not rule out the possibility that gp150 in the virion does bind to C127 cells. Lymphocytes may have a higher level in general of the gp150-His binding molecule i.e. some may be present on epithelial cells but to a lesser degree. If gp150-His has a lower affinity compared to native gp150 the level of the receptor on lymphocytes may facilitate binding at a significant level in contrast to that on epithelial cells. With the fortuitous acquisition

of pVR1255/gp150 it is highly recommended that the generation of eukaryotic expressed secreted gp150 for binding studies be pursued by modification of the pVR1255/gp150 vector in addition to further work suggested with gp150-His.

4.2.1 Neutralisation Activity of Gp150 Specific Antiserum

In order to determine the mechanism of neutralisation by the gp150 specific serum reported to neutralise MHV-68 infection, the ability of antibody to block binding of MHV-68 to cells was assessed. The anti-gp150 serum did not show inhibition of virus attachment in contrast to the anti-MHV-68 antiserum. This initial observation suggested that gp150 may be involved in a post attachment entry event e.g. fusion. For example antibodies to gH, a glycoprotein required for fusion, do not block attachment of HSV-1 to cells however they are neutralising by inhibiting fusion (Fuller *et al.*, 1989). Similarly, a monoclonal antibody to gH of EBV inhibits fusion but not attachment of virus to cells (Miller & Hutt-Fletcher, 1988). In order to pursue this neutralisation assays were set up with the intention of setting up a system to see if neutralisation could be overridden by the addition of the fusion induction agent poly ethylene glycol (Forrester *et al.*, 1992).

Neutralisation of MHV-68 infection using the anti-gp150 antibody was not observed in contrast to previous studies (Stewart *et al.*, 1996). The reported neutralising activity was described as weak with a 50% reduction in plaque number occurring at a 1/30 dilution and 84% reduction at a 1/10 dilution. It may be argued that the antiserum preparation had perhaps degraded over time however, with regards to detection of gp150 in western analysis, immunofluorescence and immunostaining the antiserum was effective. Several aliquots from the frozen stock of gp150 specific antiserum were tested. Perhaps differences in the passage number of cells used and different virus stocks influenced the outcome of these neutralisation assays. However, the same cell line, C127, was used and virus was prepared in the same way.

It should be noted that the anti-gp150 antibody was generated against a GST-gp150 fusion protein (Stewart *et al.*, 1996) consisting of a portion of i.e. not the whole extracellular domain of gp150. Failure to demonstrate neutralising activity in this circumstance does not mean gp150 is not a neutralising determinant because the antibody is against only a portion of the protein which possibly has limited neutralising epitopes and the region not included in the portion of gp150 used for the generation of the antisera may contain important neutralising epitopes. Glycoproteins that are described as neutralising determinants commonly have a limited number of neutralising epitopes, e.g. for the influenza haemagglutinin glycoprotein five main neutralising epitopes have been identified in the knob region (Wiley *et al.*, 1981). The attachment of antibodies to other regions of the molecule does not necessarily result in neutralisation. The portion of gp150 used for the generation of GST-gp150 corresponds to nucleotides 69716 to 70199 on the MHV-68 genome within gp150 (see figure 1.8 for location), a total of 161 amino acids (the entire extracellular portion is predicted to be 435 amino acids and therefore the region used in the GST-gp150 fusion protein represents approximately one third of the extracellular portion). A stretch of 66 amino acids at the N terminal and 208 at the carboxyl end of the extracellular portion are not included.

It must also be remembered that antibody effected neutralisation *in vitro* differs from that of *in vivo* where not only physical blocking of virus from adhering to cells and virus aggregation occurs but activation of complement and targeting of cells for phagocytosis is in effect to clear virus therefore weak neutralising activity *in vitro* may equate to a much stronger effect *in vivo*. The vaccination study by Stewart *et al.*, using the VV^{gp150} supports the description of gp150 as a neutralising epitope as neutralising antibodies were detected prior to MHV-68 challenge in animals vaccinated with VV^{gp150} in contrast to those which received the control VV^{gpt} (Stewart *et al.*, 1999a).

Due to the inability to detect neutralisation using the anti-gp150 antibody described here the generation of a new anti-gp150 antiserum, using the entire extracellular portion of gp150 is advisable. This could be done using the gp150-His protein as an

antigen or alternatively the recombinant vaccinia virus engineered to express gp150, VV^{gp150}. Use of VV^{gp150} for the generation of antiserum would be preferable due to the eukaryotic nature of the protein expressed. This antiserum could subsequently be used to definitively determine the ability of gp150 to induce MHV-68 neutralising antibodies. Followed by studies, in the likely event of neutralisation, into the mechanism of neutralisation e.g. whether the antibody blocks virus attachment to cells, in order to determine the role of gp150.

The effect of preincubation of virus with antisera prior to application to splenic lymphocytes was tested. Following incubation of MHV-68 with dilutions (ranging from ½ to 1/100) of either anti-MHV-68 antibody or anti-gp150 antibody increased levels of binding MHV-68 were observed. Up to 50% of lymphocytes were detected to have bound MHV-68 in contrast to the normal level of approximately 10% . This is likely to be due to the attachment of antibody coated MHV-68 virions to cells via Fc receptors expressed by lymphocytes. From a dilution of 1/500 of anti-gp150 antibody the enhanced binding was no longer seen whereas the anti-MHV-68 antibody needed to be diluted further (1/1000 onwards) for the enhanced binding effect to cease. This difference may be due to a greater number of virion epitopes recognised by the anti-MHV-68 antibody in comparison to the anti-gp150 antibody. In order to test the importance of epitopes recognised by the anti-gp150 antiserum in virus attachment to splenic lymphocytes a preparation of the Fab fragments alone is required to avoid the antibody enhancement of attachment. The results here show that high concentrations of antibody can enhance binding of MHV-68 to splenic lymphocytes. Whether this would subsequently result in enhanced infection is not known. With regards to virus infection *in vivo* physiological levels of antibody are not likely to occur at the high concentrations of antibody shown here to enhance attachment to lymphocytes.

4.2.2 Gp150 Based Vaccination

The work described here forms a preliminary study into the potential of gp150 as a DNA vaccine encoded immunogen against MHV-68 infection. The use of gp150 was intended as a model to determine the potential of a virion membrane glycoprotein in a DNA vaccine to induce protection against a gammaherpesvirus infection. In addition, the investigation provided an opportunity for familiarisation with the relatively new gene gun technology for vaccination. The feasibility of this method of vaccination in terms of the preparation and materials required could also be considered.

The main strategy was to prime animals with gp150 DNA and boost with a recombinant vaccinia virus, VV^{gp150} which expresses gp150. This strategy of DNA priming-recombinant vaccinia boosting has been shown to be highly effective at inducing specific CTL cells for HIV (Hanke *et al.*, 1999). Here, this method was used with the intention of inducing both cellular and humoral immunity against MHV-68 in order to control the primary infection and prevent the establishment of virus latency. Levels of lytic and latent virus were measured as a readout for success of protection against MHV-68. Subcutaneous administration of the recombinant vaccinia virus VV^{gp150} had previously been shown to induce MHV-68 neutralising antibodies in mice (Stewart *et al.*, 1999a). The ability of co-administration of a plasmid encoding type I alpha interferon (IFNαI) to enhance the protective effect of immunity induced by the gp150 DNA vaccine was also tested. During viral infections type I interferon is detected at high levels and evidence exists which indicates this promotes generation of $CD8^+$ T cells and in addition the survival of pre-existing memory CTLs (Tough *et al.*, 1996a & b).

Immunisation of mice with gp150 DNA followed by a boost of either gp150 DNA or either of the recombinant vaccinia viruses - VV^{gp150} or the control VV^{gpt}, resulted in a trend, although not significant, towards reduced lung titres upon subsequent challenge with MHV-68. From the data obtained here it is concluded that these vaccination strategies are not effective at limiting the acute MHV-68 infection. Significantly reduced MHV-68 lung titres (approximately 10 fold less) were detected

in groups that had been immunised with gp150 DNA + IFNαI DNA or IFNαI DNA alone. This suggests that IFNαI can promote protection non specifically. The duration of expression from the administered DNA is not known however, following intramuscular administration of vector pVR1255/IFNαI expression has been detected at least a month after introduction into animals (K Robertson, personal communication). The presence of IFNαI was determined to be systemic. Considering this and the data here, increased levels of IFNαI present during primary infection of MHV-68 may improve the efficacy of the naive hosts immune response at controlling the acute infection. High levels of IFNαI in the blood may have a direct antiviral effect and stimulate components of the innate immune response such as NK cells (Vilcek & Sen, 1996, Biron, 1998, Diefenbach *et al.*, 1998). As an adjuvant this could be unsuitable when the aim is to generate $CD8^+$ memory T cells. It is known that MHV-68 is sensitive to type I interferon (Dutia *et al.*, 1999b).

Promisingly immunisation with gp150 DNA followed by VV^{gp150} stimulated the production of MHV-68 specific antibodies prior to MHV-68. Furthermore, anti-MHV-68 antibodies were present earlier post MHV-68 challenge in recipients of two doses of gp150 DNA alone in comparison to control mice. This shows that gp150 DNA is effective at priming a specific humoral reponse and is specifically enhanced when used in conjunction with the recombinant vaccinia virus expressing gp150. A specific priming and boost are required for early generation of the MHV-68 specific antibody response i.e. the use of gp150 DNA followed by the control VV^{gpt} did not result in appearance of MHV-68 antibodies any earlier than the control mice. Antibody data for the groups receiving IFNαI DNA is not available and was not generated owing to limited materials for analysis.

The antibody response detected in all groups tested predominantly consisted of IgG2a and IgG2b isotypes indicating a T helper 1 response which does not differ from the normal response to MHV-68 infection of mice. Unfortunately an anti-IgA antibody was not available for detection of the IgA isotype population. Ideally this would have been done and is strongly recommended to be performed in repeat and further trials. Intranasal administration of the recombinant vaccinia virus aimed to

induce mucosal immunity, where IgA is the predominant isotype of antibody (Mazanec *et al.*, 1996), which could neutralise MHV-68 at the site of primary infection.

None of the vaccine regimes prevented the transient splenomegaly which occurs following MHV-68 infection. This contrasts to the marked reduction in splenomegaly observed previously in animals that were immunised subcutaneously with VV^{gp150} (Stewart *et al.*, 1999). Of note was the group that received IFNαI DNA alone which at day 59 post MHV-68 challenge had 10 times less spleen cells present compared to all other groups in which levels had returned to normal. The reason for this is not known, it may be due to the migration of splenocytes from the spleen due to prolonged expression of the introduced IFNαI DNA. IFNαI can regulate lymphocyte trafficking by inducing the expression of the L-selectin receptor (Evans *et al.*, 1993). It would be interesting to see if this phenomenon occurs in the absence of virus infection. This effect needs further investigation with regards to use of IFNαI in DNA vaccination. It suggests prolonged expression of IFNαI is undesirable. The prolonged presence of IFNαI may subdue the immune response as it is anti-proliferative.

None of the vaccination regimes prevented establishment of latency in the spleen. However, a promising result was seen at day 15 where groups which received gp150 DNA followed by a booster of either VV^{gp150} or VV^{gpt} had significantly lower (approximately 6 fold) amounts of latent virus in the spleen in comparison to the control group. The reduction in peak latent virus titre in the spleen for these groups suggests that vaccinia virus efficiently boosts the immune response to the DNA prime. The fact that either vaccinia virus produces a similar reduction in peak latent virus titre indicates that this is a non specific boost possibly accountable by a bystander effect induced by the vaccinia viral infection in general (Tough *et al.*, 1996a & b).

The reduction in peak latent viral titres in recipients of gp150 DNA + either recombinant vaccinia virus may be due to reduced virus reaching the spleen due to

enhanced antiviral response in the lung. However, no significant reduction of infectious virus in the lung was seen for these groups. Unfortunately no evidence provided here shows that the reduction in peak latent virus titres is due to a gp150 specific response. An essential component of any further studies would be the inclusion of a DNA control i.e. pVR1255 alone without the gp150 insert. This was not available for the investigation described here.

Considering the viral titre data alone it could be concluded that the gp150 based vaccine does not have a significant effect on the acute lung infection but rather produces an immune response that acts against infection of splenocytes. If due to a specific humoral response to gp150, this would tie in with the binding data showing that the recombinant gp150 protein does not bind epithelial cells whereas it displays binding activity to splenic lymphocytes. However, the level of neutralising ability of anti-gp150 antibody against MHV-68 infection of epithelial cells in comparison to B cells is not known. The mechanism of the reduction in peak splenic latency is not clear.

Although occurring earlier the overall level of MHV-68 specific antibody does not differ in the recipients of gp150 DNA and VV^{gp150} compared to control mice. The proportion of antibodies generated against gp150 is not known or the neutralising activity of the humoral response. Further investigations should include neutralisation analysis of the antibody response and the determination of the level of gp150 specific antibody. The gp150-His protein could be used in an ELISA assay to detect gp150 specific antibodies in the sera. The lack of reduction in viral lung titres detected here may be due to inadequate levels of MHV-68 neutralising antibody. During the normal course of MHV-68 infection $CD8^+$ T cells are the main component of the immune response which effects control of the acute infection. In the absence of antibody, clearance of the acute lung infection is slightly delayed (Ehtisham *et al.*, 1993). It is not known whether increased levels of antibody against MHV-68 in general and gp150 specifically would provide complete protection or at least a significant decrease in infectious lung titres. To test this passive transfer of MHV-68 or gp150 specific antisera to naïve mice could be done prior to challenge.

This preliminary vaccination experiment needs to be repeated to confirm the effects seen. It would be worth concentrating on the lung at early times post infection e.g. days 1, 3, 5, 6 and 8. This is suggested as there may be significant differences between the groups in the acute infection not detected in the study described here due to the limited time points analysed. It would also be worth comparing the efficacy of gp150 DNA vaccination administered using the gene gun in comparison to an alternative route such as intramuscular injection. Although with intramuscular vaccination more plasmid (over 100 fold) is required generation of gene gun bullets is time consuming and expensive. The production of the gene gun bullets takes time to master and the coating process (where the gold microparticles onto which DNA has been precipitated are coated onto the inside of a tube) is inefficient and can result in much wastage.

Analysis of the CTL response generated by this method of vaccination ought to be addressed. In the case of this pilot study this was not done due to lack of a comprehensive CTL analysis system, preferably further investigations should include the analysis of MHV-68 specific CTLs, identification of the major epitopes they recognise, their activity and the overall activation of memory T cells.

A notable feature of MHV-68 is that no matter what level of infectious virus titre occurs in the lung, splenic latency is established. Whatever the level of peak latent virus titre in the spleen long term latency settles at a common low level. The inclusion of latency associated antigens in addition to lytic antigens into a vaccine is likely to be pursued due to the difficulty in preventing the initial infection and the establishment of latency.

Control of the acute lung infection in order to prevent spread of virus may demand a level of neutralising antibody not elicited by the vaccination strategy used here. Infection of a B cell in the lung may result in rapid trafficking of the virus from the lung to other sites such as the spleen. It seems logical that if a huge reduction in virus titre in the lung could be achieved via neutralisation by antibody and elimination of lytically infected cells by $CD8^+$ T cells the amount of virus available

to encounter trafficking B cells would be reduced reducing the probability of establishment of latency. Whether an additional vaccine boost of gp150 DNA or VV^{gp150} might effect protection remains to be tested. As sterilising immunity for the acute infection seems unlikely addition of vaccine components to generate specific immunity to latency associated antigens may prove effective. The lack of significant reduction in the lung viral titres using the vaccination strategy described here may be due to the induction of insufficient levels of neutralising antibodies and MHV-68 $CD8^+$ T cells. Use of a protein vaccine in combination with an adjuvant might prove more effective at eliciting protection against MHV-68 infection. However, a preliminary study using gp150-His as an immunogen suggests this is not the case (K Robertson, personal communication). In addition the use of a protein immunogen does not efficiently target the cell mediated immunity in contrast to DNA vaccination and the use of vaccinia virus recombinants both of which target the immunogen to the MHCI pathway for presentation to CTLs.

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