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**The generation of a
herpes simplex virus vector
to target motor neurons**

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the requirements of the degree of Doctor of Philosophy

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List of Contents

1. INTRODUCTION	1
1.1 Virion structure.....	1
1.1.1 Core	1
1.1.2 Capsid.....	2
1.1.3 Tegument.....	2
1.1.4 Envelope.....	3
1.2 Structure and organization of the HSV genome.....	3
1.2.1 The <i>a</i> sequence	4
1.3 Pathogenesis of HSV.....	6
1.4 Lytic life cycle.....	7
1.4.1 Gene expression.....	8
1.5 HSV latent infection.....	11
1.5.1 Latency-Associated Transcripts (LATs)	11
1.5.2 Latency promoters	13
1.5.3 Reactivation.....	13
1.6 HSV-1 Adsorption and Penetration.....	14
1.7 HSV-1 Egress	15
1.8 Adsorption.....	17
1.8.1 Glycoproteins involved	17
1.8.2 Proteoglycans	18
1.8.3 Heparan sulphate glycosaminoglycans.....	18
1.9 Penetration.....	19
1.10 Glycoprotein C (gC).....	20
1.10.1 Oligomeric structure.....	20
1.10.2 HS binding domains	21
1.10.3 gC null mutant viruses.....	22
1.11 Glycoprotein B (gB).....	23
1.11.1 Role in Adsorption and Penetration	24
1.11.2 HS binding domains	24
1.11.3 gD and its receptors.....	24
1.12 Other HSV binding ligands	25
1.13 HS binding domain-deleted virus.....	28
1.14 HSV entry into primary neurons	29
1.15 Cell tropism and serotype differences in viral entry	30
1.16 Motor neuron infection by HSV-1	32
1.17 ICP34.5.....	32
1.18 Viral vectors for gene therapy.....	34
1.18.1 Viral vectors for CNS applications.....	35
1.18.2 Herpes simplex virus vectors.....	36
1.19 Cell culture models.....	38
1.19.1 NSC-19 and NSC-34 cell lines.....	39
1.20 Gangliosides	40
1.21 Escherichia coli heat-labile enterotoxin (LT).....	43

1.21.1	Genetics	43
1.21.2	Structure	43
1.21.3	Mode of action.....	44
1.21.4	Binding of the B pentamer	45
1.21.5	Binding specificities of LTB	46
1.21.6	LTB fusion proteins.....	46
1.22	Translational differences between prokaryotes and eukaryotes	47
1.22.1	Selection of start sites in prokaryotic mRNAs	47
1.22.2	The Shine-Dalgarno (SD) interaction.....	48
1.22.3	Selection of start sites in eukaryotic mRNAs.....	49
1.22.4	Alternative mechanisms	50
1.22.4.1	Leaky scanning	50
1.22.4.2	Reinitiation	51
1.22.4.3	Internal initiation of translation	51
1.23	Project rationale.....	51
2.	MATERIALS.....	53
2.1	Bacteria.....	53
2.2	Plasmids.....	53
2.3	Cells and Tissue Culture Media	53
2.3.1	ETC10.....	53
2.3.2	DMEM10.....	53
2.4	Viruses	54
2.4.1	Strain 17 ⁺	54
2.4.2	Mutant gC-ve.....	54
2.4.3	Mutant 1716.....	54
2.5	Antisera.....	54
2.6	Radiochemicals.....	54
2.7	Enzymes	55
2.8	Sequencing	55
2.9	Solutions.....	55
2.9.1	Bacterial Culture Reagents	55
2.9.2	Tissue Culture Reagents	56
2.9.3	Agarose Gel Reagents	56
2.9.4	Southern blot Reagents.....	56
2.9.5	SDS-PAGE Reagents	57
2.9.6	Non-denaturing PAGE Reagents.....	57
2.9.7	Western Blot Reagents	57
2.10	Chemicals	58
2.11	Other materials	58
2.11.1	Centrifuges	58
2.11.2	Film.....	58
2.11.3	Plasticware.....	58
2.11.4	Miscellaneous Equipment	58
3.	METHODS.....	59
3.1	Tissue Culture.....	59
3.2	Cells.....	59

3.3	BHK21/C13 Cells.....	59
3.4	3T6 Cells	59
3.5	NSC-19 and NSC-34	59
3.6	Passaging BHK and 3T6 Cells	60
3.7	Passaging NSC-19 and NSC-34 Cells.....	60
3.8	Cryopreservation of BHK and 3T6 Cells.....	60
3.9	Cryopreservation of NSC-19 and NSC-34 Cells.....	60
3.10	Growth and harvest of HSV	61
3.11	Titration of virus stocks.....	61
3.12	Preparation of HSV DNA.....	62
3.13	One-cycle Replication Kinetics.....	62
3.14	Large scale isolation of plasmid DNA: Promega © Wizard™ Maxiprep.....	63
3.15	Restriction endonuclease digestion	64
3.16	Phenol chloroform extraction and precipitation of plasmid DNA	64
3.17	Ligation of plasmid DNA.....	64
3.18	Transformation of E. coli	64
3.19	Small scale preparation of plasmid DNA.....	65
3.20	Agarose gel electrophoresis.....	66
3.21	Minigel electrophoresis	66
3.22	Elution of Restriction Enzyme Fragments from Agarose Gel.....	66
3.23	Transfection of virus DNA by CaPO ₄ and DMSO boost.....	67
3.24	Transfection of mammalian cells	67
3.25	Plaque purification.....	68
3.26	DNA Extraction of HSV Plaque Isolates	68
3.27	Radiolabelling DNA	69
3.28	Southern Blot and Hybridisation.....	69
3.29	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)...	70
3.30	Non-denaturing Polyacrylamide Gel Electrophoresis.....	70
3.31	Western Blotting.....	71
3.32	Protein Expression in Infected Cell Extracts.....	71
3.33	Harvesting Protein Samples for Denaturing Gels	72
3.34	Concentration of Protein Samples	72
3.35	Harvesting Protein Samples for Non-denaturing Gels	72
3.36	Indirect Immunofluorescence	73
3.37	X-gal Staining.....	73
3.38	Viral Adsorption to Cells.....	74
3.39	HSV Glycolipid-Binding ELISA	74
3.40	Heparin-binding ELISA	75
3.41	In vitro Transcription.....	75
4.	CONSTRUCTION OF HSV-1 RECOMBINANT VIRUSES CONTAINING	
	LTB-gC GENE FUSIONS	78
4.1	Introduction	78
4.2	Construction of Mutant Viruses: RFa and RFb.....	79
4.3	Subcloning of etxB	79
4.4	Creation of gC-LTB fusions.....	79
4.4.1	PCR of gC(a).....	82

4.4.2	PCR of gC(b).....	87
4.5	Creation of an expression plasmid	87
4.5.1	Subcloning of the CMV IE promoter	92
4.5.2	Subcloning of gC(a)-LTB into RL1.dCMV	92
4.5.3	Subcloning of gC(b)-LTB into RL1.dCMV	104
4.5.4	Subcloning of a PolyA site into the expression plasmids.....	104
4.5.5	Sequencing of pRFa and pRF.....	110
4.6	Co-transfection of pRFa/pRFb with HSV-1 gC-ve DNA	114
4.6.1	Generation of RFa and RFb.....	114
4.7	Conclusion	119
5.	CHARACTERIZATION OF RFa & RFb	121
5.1	Introduction	121
5.2.	gC Expression.....	121
5.3	ICP34.5 Expression	122
5.4	LTB-gC Gene Expression in RFb	122
5.4.1	Western Blotting.....	122
5.4.2	Indirect Immunofluorescence	133
5.5	LTB-gC gene expression in RFa	133
5.6	Transfection of pRFa and pRFb	133
5.6.1	Western Blot Analysis	137
5.6.2	Indirect Immunofluorescence	137
5.7	In vitro transcription of LTB.gCb gene fusion.....	137
5.8	In vitro Replication of RFb.....	137
5.9	In vivo Adsorption of RFb	138
5.10	In vitro Adsorption of RFb	151
5.11	Conclusion	155
6.	FINAL CONCLUSION & DISCUSSION.....	161
6.1	Position of research approach relative to gene therapy strategies for neurological disease	167
7.	REFERENCES	171

List of Figures

Figure 1.1	Gross organisation of the HSV-1 genome.....	8
Figure 1.2	HSV-1 Gene Regulation.....	42
Figure 1.3	Structure of gangliosides and related compounds.....	42
Figure 4.1	Schematic overview of cloning steps (part 1).....	80
Figure 4.1	Schematic overview of cloning steps (part 2).....	81
Figure 4.2	Schematic subcloning of <i>extB</i> into pGEM2fz(+)	83
Figure 4.3	Schematic diagram of gC(a) PCR	84
Figure 4.4	PCR-amplification of gC(a)	84
Figure 4.5	Schematic cloning of gC(a) PCR product into pGEM.LTB.....	85
Figure 4.6	Cloning of gC(a) into pGEM.LTB	86
Figure 4.7	Schematic diagram of gC(b) PCR	88
Figure 4.8	PCR-amplification of gC(b)	89
Figure 4.9	Schematic diagram of gC(b) cloning into pGEM.LTB	90
Figure 4.10	Cloning of gC(b) into pGEM.LTB.....	91
Figure 4.11	Schematic construction of RL1.del	93
Figure 4.12	Subcloning of the CMV IE promoter into RL1.del	94
Figure 4.13	Subcloning of the CMV IE promoter	95
Figure 4.14	Subcloning of the CMV IE promoter into RL1.del	96
Figure 4.15	Schematic diagram of LTB-gC(a) PCR	97
Figure 4.16	PCR-amplification of LTB-gC(a).....	98
Figure 4.17	Schematic diagram of LTBgC(a) cloning into RL1.dCMV.....	99
Figure 4.18	Cloning of LTB-gC(a) into RL1.dCMV	100
Figure 4.19	Schematic drawing of LTB-gC(b) PCR	101
Figure 4.20	PCR-amplification of LTB-gC(b)	101
Figure 4.21	Schematic diagram of LTBgC(b) cloning into RL1.dCMV.....	102
Figure 4.22	Cloning of LTB-gC(b) into RL1.dCMV	105
Figure 4.23	Subcloning of the HSV-2 IE5 polyadenylation sequence	105
Figure 4.24	Schematic diagram of subcloning of PolyA into RL1.dCMV.LTBgCa	106
Figure 4.25	Cloning of PolyA into RL1.dCMV.LTB-gC(a)	107
Figure 4.26	Schematic diagram of subcloning of PolyA into RL1.dCMV.LTBgCb ...	108
Figure 4.27	Cloning of PolyA into RL1.dCMV.LTB-gC(b)	109
Figure 4.28	Schematic diagram of <i>EcoRI</i> -digested HSV-1.....	115
Figure 4.29	Schematic representation of recombinant viruses	116
Figure 4.30	Schematic diagram of Southern blotting bands for gC-ve, RFa and RFb using a RL1.del probe.....	117
Figure 4.31	Southern blot of purified RFb and partially purifiedRFa.....	118
Figure 5.1	Western blot using anti-gC antisera	123
Figure 5.2	Western blot using 137 anti-ICP34.5 antisera	124
Figure 5.3	Western blot of RFb using 118-8	126
Figure 5.4	Western blot of RFb using 118-8	127
Figure 5.5	Western blot using Z1F11 antibody	128
Figure 5.6	Coomassie Blue-Stained SDS-PAGE Gel of RFb viral proteins	129
Figure 5.7	Western blot of 118-8 Sensitivity.....	130
Figure 5.8	LDS47 Sensitivity Assay.....	131
Figure 5.9	LDS102 Sensitivity Assay.....	132

Figure 5.10 RFb-infected BHKs stained with 118-8 and LDS102	134
Figure 5.11 Western blot of partially purified RFa against 118-8	135
Figure 5.12 Western blot of RFa using LDS102	136
Figure 5.13 Western blot of transfected cell extracts using 118-8.....	139
Figure 5.14 Western blot of transfected cell extracts using LDS102	140
Figure 5.15 <i>In vitro</i> transcription of pGEM.LTB.gC(b)	141
Figure 5.16 <i>In vitro</i> growth of 17 ⁺ , 1716, gC-ve and RFb in BHK cells.....	142
Figure 5.17 <i>In vitro</i> growth of 17 ⁺ , 1716, gC-ve and RFb in 3T6 cells.....	143
Figure 5.18 <i>In vitro</i> growth of 17 ⁺ , 1716, gC-ve and RFb in NSC-19 cells	144
Figure 5.19 <i>In vitro</i> growth of 17 ⁺ , 1716, gC-ve and RFb in NSC-34 cells	145
Figure 5.20 <i>In vivo</i> adsorption of 17 ⁺ , gC-ve and RFb to BHK cells	147
Figure 5.21 <i>In vivo</i> adsorption of 17 ⁺ , gC-ve and RFb to NSC-19 cells	148
Figure 5.22 <i>In vivo</i> adsorption of 17 ⁺ , gC-ve and RFb to NSC-34 cells	149
Figure 5.23 <i>In vivo</i> adsorption of 17 ⁺ , gC-ve and RFb to Heparan	152
Figure 5.24 <i>In vitro</i> adsorption of 17 ⁺ , gC-ve and RFb to Gangliosides	153
Figure 5.25 Ganglioside structures	158

List of Tables

Table 1. 3T6, BHK, NSC-19 and NSC-34 titres for single cycle growth kinetics.....	146
Table 2. Titres of Adsorption Studies on BHK, NSC-19, and NSC-34 cells.....	150
Table 3. % normalised binding with respect to heparan binding	154

ABBREVIATIONS

Chemicals and Compounds

Amp	ampicillin
APS	ammonium persulphate
BSA	bovine serum albumin
CaPO ₄	calcium phosphate
CS	chondroitin sulphate
CT	colera toxin
C ₂ H ₄ O ₂	acetic acid
dH ₂ O	distilled water
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DS	dermatan sulphate
EDTA	ethylenediaminetetra acetic acid
EtBr	ethidium bromide
EtOH	ethanol
GAG	glycosaminoglycan
GlcA	glucuronic acid
HCl	hydrochloric acid
HS	heparan sulphate
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HRP	horseradish peroxidase
H ₂ SO ₄	sulphuric acid
IdoA	iduronic acid
IMP	integral membrane protein
KAc	potassium acetate
LT	<i>E. coli</i> heat-labile enterotoxin
MeOH	methanol

MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
mRNA	messenger ribonucleic acid
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
NP40	Nonidet P-40
PBS	phosphate buffer saline
RNA	ribonucleic acid
RnaseA	Ribonuclease A
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' –tetramethylethylene diamine
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
X-gal	5-chloro-4-bromo-3-indoyl-2-D-galactoside

Descriptions

Ab	polyclonal antibody
cpe	cytopathic effect
ECM	extracellular matrix
MAb	monoclonal antibody
oligo	oligonucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pi	post infection
RT	room temperature
UV	ultra violet light

Measurements

°C	degrees Celcius
Ci	Curie
d	day
g	force of gravity
h	hour
M	molar
mA	milliamp
mg	milligram
min	minute
ml	millilitre
mM	millimolar
moi	multiplicity of infection
Mr	molecular weight
ng	nanogram
nm	nanometre
OD	optical density
pfu	plaque forming units
rpm	revolutions per minute
s	second
μl	microlitre
μm	micrometer
U	unit(s)
V	voltage
v/v	volume/volume (ratio)
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

Tissue Culture

DMEM10	Dulbecco's modified eagles medium with 10% foetal calf serum
ETC10	Eagle's media with 10% newborn calf serum
FCS	foetal calf serum
NCS	newborn calf serum

Amino Acids (single and three letter code)

Ala	A	Alanine	Arg	R	Arginine	Asn	N	Asparagine
Asp	D	Aspartic Acid	Cys	C	Cysteine	Gln	Q	Glutamine
Glu	E	Glutamic Acid	Gly	G	Glycine	His	H	Histidine
Ile	I	Isoleucine	Leu	L	Leucine	Lys	K	Lysine
Met	M	Methionine	Phe	F	Phenylalanine	Pro	P	Proline
Ser	S	Serine	Thr	T	Threonine	Trp	W	Tryptophan
Tyr	Y	Tyrosine	Val	V	Valine			

Genetic code

Second Position

		Second Position				
		U	C	A	G	
First Position (5' end)	U	UUU } Phe	UCU } Ser	UAU } Tyr	UGU } Cys	U
		UUC } Phe	UCC } Ser	UAC } Tyr	UGC } Cys	C
		UUA } Leu	UCA } Ser	UAA } Stop	UGA } Stop	A
		UUG } Leu	UCG } Ser	UAG } Stop	UGG } Trp	G
	C	CUU } Leu	CCU } Pro	CAU } His	CGU } Arg	U
		CUC } Leu	CCC } Pro	CAC } His	CGC } Arg	C
		CUA } Leu	CCA } Pro	CAA } Gln	CGA } Arg	A
		CUG } Leu	CCG } Pro	CAG } Gln	CGG } Arg	G
	A	AUU } Ile	ACU } Thr	AAU } Asn	AGU } Ser	U
		AUA } Ile	ACC } Thr	AAC } Asn	AGC } Ser	C
		AUA } Ile	ACA } Thr	AAA } Lys	AGA } Arg	A
		AUG } Met	ACG } Thr	AAG } Lys	AGG } Arg	G
	G	GUU } Val	GCU } Ala	GAU } Asp	GGU } Gly	U
		GUC } Val	GCC } Ala	GAC } Asp	GGC } Gly	C
		GUA } Val	GCA } Ala	GAA } Glu	GGA } Gly	A
		GUC } Val	GCG } Ala	GAG } Glu	GGG } Gly	G
		Third Position (3' end)				

Abstract

Herpes simplex virus (HSV) is a neurovirulent virus that in the course of natural infection of man predominantly infects sensory neurons. The aim of this project was to develop a safe, nonvirulent HSV, capable of expressing exogenous genes which altered the binding characteristics of the virus so that tropism was directed predominantly to motor nerves. It was envisaged that these viruses could then act as prototypes for gene therapy vectors targeted to the treatment of motor nerve diseases.

To achieve this, two mutant viruses were created, RFa and RFb. These contained deletions of the main HSV glycoprotein involved in cellular binding (glycoprotein C). Gene fusions were created of truncated portions of gC (amino acids 377-511(RFa) and amino acids 477-511(RFb)) to E.coli heat-labile enterotoxin B-subunit (LTB). The gene fusions were inserted in the RL1 gene thereby abolishing expression of the virulence factor ICP34.5. LTB is a ligand which binds to several gangliosides, including GM1 and GM2 which are motor neuron markers. It was hoped that by deletion of the main viral protein involved in adsorption to cells and replacing it with an LTB-containing fusion protein, the tropism of the mutant viruses could be altered to promote an increase in motor neuron infection.

RFb was constructed. RFa was constructed but could not be purified to homogeneity. This was thought to be due to poor adsorption/penetration or cell-to-cell spread, brought about by expression of the LTB fusion protein. RFb was analysed to determine the effect of expression of the novel LTB fusion protein within the context of the HSV genome. Western blot analysis using antibodies directed against LTB failed to detect expression of the LTB-gC fusion protein. In vitro replication studies showed that the RFb was non-virulent as demonstrated by its inability to replicate in growth arrested 3T6 cells, a phenotype characteristic of HSV which fails to produce ICP34.5. However no marked difference in virus replication kinetics was seen between RFb and wild type HSV (17+) on two motor neuron-like cell lines (NSC-19 and NSC-34). *In vitro* and *in vivo* adsorption studies were also carried out on the mutant virus. Again, no increased

adsorption was seen with respect to wild type virus on a variety of cell lines and to a variety of gangliosides as assayed by ELISA techniques. Interestingly, the role for gC in binding to several gangliosides was seen. The affinity of wild type virus for several individual gangliosides was shown to be greater than that of gC-null HSV. *In vitro* wild type HSV bound more strongly to several gangliosides including GD3 and GT1b than it did to heparin, the main HSV binding ligand. This finding opens the possibility of investigating whether the inherent neurotropism of HSV may be related to specific ganglioside binding mediated through the glycoprotein gC.

1. INTRODUCTION

Herpes simplex virus (HSV) is a member of the family Herpesviridae. This family includes many viruses of medical and veterinary importance. Herpes simplex virus types 1 and 2 belong to the subfamily Alpha herpesvirinae, as they have the capacity to establish latent infections primarily in the sensory ganglia. HSV is a neurotrophic virus responsible for a broad spectrum of clinical diseases ranging from relatively benign cutaneous lesions to fatal encephalitis. The life cycle of HSV includes acute, latent and recurrent phases. In acute infection, the genes of HSV are expressed and the extent of the disease is controlled by the cellular and humoral responses of the host. In latent infection, gene expression is repressed except for a limited region of the HSV genome encoding latency-associated transcripts (LATs). The virus can remain in this latent state in humans for life without destroying the sensory neurons in which it resides. Latent virus can be induced by a variety of stimuli to reactivate from this quiescent state to cause a recurrent peripheral infection.

1.1 Virion structure

Herpes viruses all have a similar virion structure which has led to their classification into one family. HSV virions are 150-200 nm in size and consist of four basic morphological elements: a nuclear core covered by an icosahedral nucleocapsid, an amorphous tegument surrounding the capsid and an outer envelope (reviewed by Rixon, 1993).

1.1.1 Core

The core of a mature HSV virion contains the viral DNA. The HSV DNA genome is approximately 152 kbp in size. The genome consists of two covalently linked components, namely, the unique long (U_L) and unique short (U_S) each of which is flanked by terminal inverted repeats (TR_L/IR_L , and TR_S/IR_S respectively). The HSV genome encodes at least 84 gene products (Roizman and Sears, 1996).

1.1.2 Capsid

Capsids are assembled in the nucleus. They are composed of protein, and are approximately 125nm in diameter, exhibiting 5:3:2 axial symmetry and comprise 162 capsomeres, of which 150 are hexameric (hexons) and 12 pentameric (pentons) and 320 triplexes that provide intercapsomeric connections (Wildy *et al.*, 1960; Scharge *et al.*, 1989; Zhou *et al.*, 1994). Three capsid forms have been observed and purified from wild type virus-infected cells. They are visualised as light scattering bands in sucrose gradients and are designated A, B and C in order of increasing distance sedimented (Gibson and Roizman, 1972). Type A capsids are empty capsids which lack viral DNA; Type B capsids also lack viral DNA, but possess 2 proteins not found in A capsids (VP21 and VP22a); Type C capsids contain viral genomes and mature into infectious virions. The capsid is composed of nine proteins:

VP23 (UL18), VP5 (UL19), VP21 (UL26), VP22a (UL26.5), VP26 (UL35), VP19C (UL38), VP11+12 (UL46), VP 13+14 (UL47), VP22 (UL49)

Capsids are shaped like hexagonal prisms with a hollow tube through the length of the longitudinal axis (Wildy *et al.*, 1960). VP5 is the major capsid protein, forming both hexons and pentons. VP22a present only in B capsids, occupies the inner space and functions as a scaffold for the icosahedral capsid shell. VP24 is a protease encoded by UL26. VP26 is a protein expressed late in the infectious cycle after the onset of DNA replication and has been shown to be present in multiple phosphorylated forms (McNabb and Courtney, 1992). This is the only capsid protein that is not required for replication in cell culture however infectious virus yield of VP26-deleted virus is decreased two-fold relative to wild type (Desai *et al.*, 1998). VP26 is important however for virus replication *in vivo* (Desai *et al.*, 1998).

1.1.3 Tegument

The tegument is an electron-dense material located between the capsid and the envelope. The amount of tegument in each virion is not equivalent. The tegument can assemble into a stable structure without capsid interaction, and its assembly or dissociation depends on

the phosphorylation state of its structural proteins (Leslie *et al.*, 1996; Morrison *et al.*, 1998). It is composed of at least 20 distinct viral proteins, some present in major, other in quite minor amounts (McGeoch *et al.*, 1993). Several tegument proteins are essential for particle formation e.g. UL48, UL36 (McGeoch *et al.*, 1993; Campbell *et al.*, 1984), while others are known to be dispensible e.g. UL46, UL47 (Zhang and McKnight, 1993). Tegument proteins are the first to encounter the intracellular environment and many of these are released into the newly infected cell. While the function of many of these proteins has yet to be precisely defined, several have been shown to aid in the initiation of the viral replicative cycle (Roizman and Sears, 1996). Among these are VP16 and the virion host shutoff protein (vhs). VP16 serves multiple functions during HSV infection: it activates transcription of the viral IE genes, binds to vhs and downregulates its activity, and forms a complex with the tegument protein VP22 (Elliot *et al.*, 1995; Lam *et al.*, 1996; O'Hare, 1993). Vhs is a tegument protein that triggers shutoff of host protein synthesis and accelerated degradation of both cellular and viral mRNAs (Fenwick and Everett, 1990).

1.1.4 Envelope

The tegument is completely enclosed in a trilaminar membrane envelope (Wildy *et al.*, 1960). This is the outermost covering of the virus and is composed primarily of lipids derived from the host cell membrane, into which are inserted at least 11 virus-encoded glycoproteins. These glycoproteins are responsible for attachment and entry of the infectious viral particle into the host cell and for cell-to-cell spread. The diameter of the envelope is approximately 150-200nm, although the exact dimensions depend on the visualisation technique.

1.2 Structure and organization of the HSV genome

HSV-1 DNA is linear, double-stranded (Becker *et al.*, 1968) and contains approximately 152 kbp (McGeoch *et al.*, 1986; Perry and McGeoch, 1988). The genomes of HSV-1 and HSV-2 share approximately 50% homology, with HSV-2 having a slightly higher G+C content (68.3% compared to 67%) (Kieff *et al.*, 1972; Davison and Wilkie, 1983;

McGeoch *et al.*, 1988a). The coding sequences of corresponding genes show in general 70-80% identity, with one major exception: the coding sequence of HSV-2 US4 (gG-2) contains an extra sequence of 1460bp (McGeoch *et al.*, 1987).

The HSV-1 and HSV-2 genomes consist of two covalently joined segments, designated long (L) and short (S) (Figure 1.1). Each component is composed of a unique sequence (U_L , U_S) flanked by terminal and inverted repeat sequences (TR, IR). The repeats of the L-component TR_L and IR_L are respectively designated $a b$ and $a' b'$, while those of the S component, TR_S and IR_S , are respectively designated $a' c'$ and $c a$ (Wadsworth *et al.*, 1975).

The L and S genome components invert relative to one another giving four equimolar isomers, termed P(prototype), I_L (inversion of the L component), I_S (inversion of the S component) and I_{SL} (inversion of both S and L components) (Sheldrick and Berthelot, 1974; Hayward *et al.*, 1975; Clements *et al.*, 1976; Wilkie and Cortini, 1976; Roizman, 1979). Due to isomerisation, restriction analysis of DNA yields three classes of fragments (Clements *et al.*, 1976; Skare and Summers, 1977) which occur at different frequencies. Fragments derived entirely from the U_L and U_S regions appear in 1M quantities relative to the molarity of intact viral DNA. As each terminus is present in only 2 of the 4 isomers, terminal sequence fragments are present in 0.5M quantities while fragments which consist of the joint sequences are present in only 1 of the 4 isomers so are in 0.25M quantities (Wilkie and Cortini, 1976; Skare and Summers, 1977).

1.2.1 The a sequence

The a sequence is a sequence that is found as a direct repeat at TR_L and TR_S and present as an inverted repeat at the L/S junction in the HSV-1 and HSV-2 genome. The a sequence is approximately 500 bp in HSV-1 (strain F), but its size varies from strain to strain. The HSV-1 (F) a sequence has a 20 bp direct repeat (DR_1), a 65 bp sequence (U_b), a 21 bp sequence (DR_2), a 37 bp sequence (DR_4), a 53 bp unique sequence (U_c) and another copy of DR_1 (Roizman and Sears, 1990). The number of a sequences at the L/S junction and at the L terminus of the DNA varies from 1 up to 10, but only a single copy

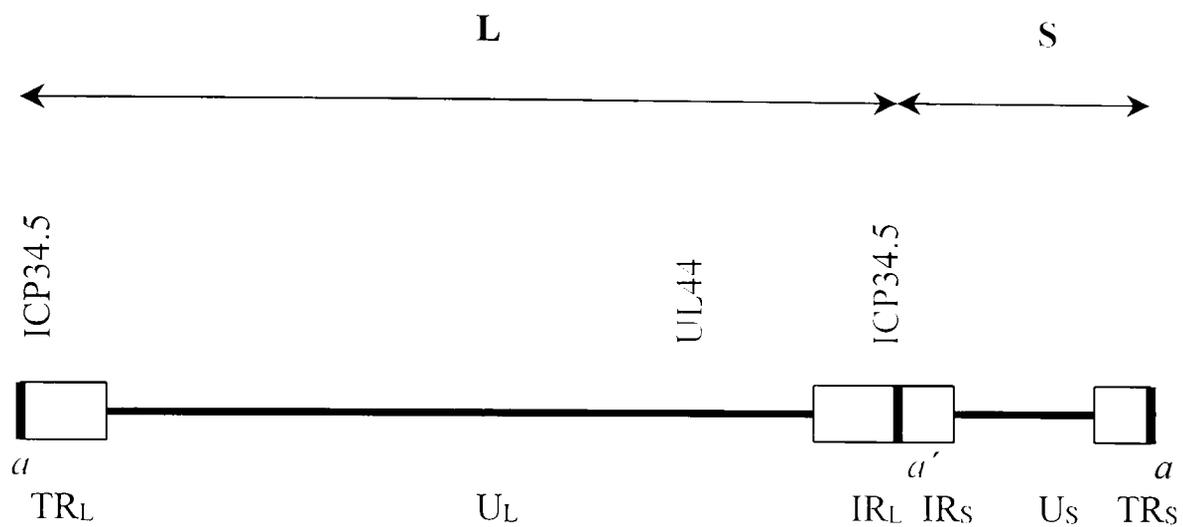


Figure 1.1 Gross organisation of the HSV-1 genome.

A conventional representation of the HSV-1 genome is shown, with unique sequences (U_L and U_S) as solid lines and major repeat elements (TR_L and IR_L, IR_S and TR_S) as open boxes. The locations of the L and S segments are marked. Terminal α sequences and the internal, opposite orientation α' sequence are indicated. Also shown are the location of three genes important in this study are shown: RL1 (gene product: ICP34.5) and UL44 (gene product: gC).

is found at the S terminus (Wilkie 1976; Wagner and Summers, 1978). The size of the *a* sequence varies from strain to strain due to the variation in the number of copies of DR₂ and DR₄. The *a* sequence appears to be a *cis*-acting site for inversion as insertion of the *a* sequence elsewhere in the genome (Mocarski *et al.*, 1980) or deletion of the entire internal inverted repeat sequences (*a' b' c'*) leads to additional inversions or the loss of the ability of the L and S components to invert respectively (Poffenberger *et al.*, 1983). Chou and Roizman (1985) demonstrated that deletion of DR4 drastically reduces inversion while deletion of both DR2 and DR4 completely abolishes inversion. Deletion of the Ub and Uc domains does not affect the ability of the *a* sequence to mediate inversion. The *a* sequence was also shown to contain the *cis*-acting sites for the circularisation of the genome after infection, for cleavage of the HSV genome into unit length concatamers and for the encapsulation of DNA (Mocarski and Roizman, 1982; Vlazny *et al.*, 1982; Varmuza and Smiley, 1985)

1.3 Pathogenesis of HSV

From studies in experimental animals and observations of human infections, a “classical theory” of HSV pathogenesis has evolved (Wildy *et al.*, 1982). According to this theory there are four stages which characterize an HSV infection:

1. Entry into the host at the time of infection, and here the virus replicates at peripheral sites such as the eyes, skin or mucosae.
2. Spread to the axonal terminae of sensory neurons is followed by retrograde intra-axonal transport to neuronal cell bodies in sensory ganglia, where further viral replication may occur.
3. Establishment of latency then occurs and lytic gene expression is repressed. At this stage no infectious virus can be detected but the viral genome remains in the neuron in a transcriptionally active state.

4. Reactivation can occur when poorly defined stimuli, e.g. stress, menstruation, sunlight cause the controls responsible for maintaining latency to break down. This leads to the production of infectious virus in the ganglion followed by anterograde transport to the periphery where, following further replication, lesions may occur at or near the site of primary infection.

1.4 Lytic life cycle

Virus binds to heparan sulphate components of the cell surface, using several glycoproteins (gC and gB). Several glycoproteins in the virion envelope are then required for penetration after initial attachment (gB, gD, gH and gL) (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992). Following attachment, the virus penetrates the cell by fusion of the virus envelope with the cell plasma membrane (Spear, 1993). Virus capsids are then transported to the nuclear pore (Tognon *et al.*, 1981; Batterson *et al.*, 1983) and DNA is released into the nucleus to allow gene expression. Transport to the nuclear pores is thought to be mediated by the cellular cytoskeleton (Kristensson *et al.*, 1986). To promote their replication, HSV manipulates the host cell such that viral proteins are preferentially synthesised, at the expense of host cell gene expression (Zelus *et al.*, 1996). Within 3 hours post-infection, HSV-DNA replication is detected in the nucleus (Roizman *et al.*, 1963; Roizman and Roane, 1964), and host protein synthesis and mRNA levels decline by approximately 90% (Zelus *et al.*, 1996). vhs is a tegument protein that triggers shutoff of host protein synthesis and accelerated degradation of both cellular and viral mRNAs (Zelus *et al.*, 1996). Its mechanism of action is unknown, but evidence suggests it is either an endo-RNase or a required subunit of an endo-RNase that also includes one or more cellular subunits (Elgadi *et al.*, 1999; Elgadi and Smiley, 1999). VP16, an abundant 65kDa virion phosphoprotein is thought to downregulate vhs activity at intermediate and late times postinfection, thereby allowing the maintenance of viral protein synthesis (Lam *et al.*, 1996).

1.4.1 Gene expression

Gene expression involves regulatory loops controlled by signals which act either in *cis* or in *trans* (Fig1.2). According to the manner in which they are expressed, viral genes can be divided into three temporal classes: immediate-early, early and late (Hones and Roizman, 1974). The polypeptides these produce are classified as α (immediate-early), β (early), and γ (late) (Roizman, 1978). Broadly speaking, α gene products function in the synthesis of β proteins, β proteins function in viral DNA replication, and γ proteins are the structural proteins of the virus. Soon after infection of the cell, the cascade of viral gene expression is initiated and it is enhanced by a viral structural protein VP16, which is brought into the nucleus with the viral genome (Campbell *et al.*, 1984; Pellett *et al.*, 1985a). VP16 transactivates immediate early genes as part of a complex with the cellular transcription factor oct-1 and other proteins (Stern *et al.*, 1989). Immediate early gene products then activate early gene expression with the resultant initiation of HSV DNA replication at the viral origins (Vlazny and Frenkel, 1981; Stow, 1982, Boehmer and Lehman, 1997)

HSV DNA replicates by a rolling circle mechanism (Ben-Porat and Towazewski, 1977; Jacob *et al.*, 1979; Roizman, 1979) with a replicative intermediate consisting of linear head-to-tail concatamers of the viral genome (Jacobs *et al.*, 1979). Synthesis is initially restricted within the nucleus to a few well-defined sites called replication compartments or inclusions (Quinlan *et al.*, 1984; Kops and Knipe, 1994) which are organized in the nuclear interior rather than at the periphery (Kops and Knipe, 1994). The number and size of these compartments increases throughout infection until the entire nucleus is filled with replicating viral DNA (Rixon *et al.*, 1983).

Three origins of replication have been identified: the origin of replication in the long region, oriL, is located in the middle of the long unique segment, between the divergent transcripts for the DNA polymerase (UL30) and the major DNA binding protein (UL29) (Quinn *et al.*, 1985); the origin of replication in the short region, oriS, is present in two identical copies in the intact genome, one in IR_S and one in TR_S (Stow, 1982). Sequence analysis revealed palindromic organisation of oriL and oriS. OriL exhibits a single

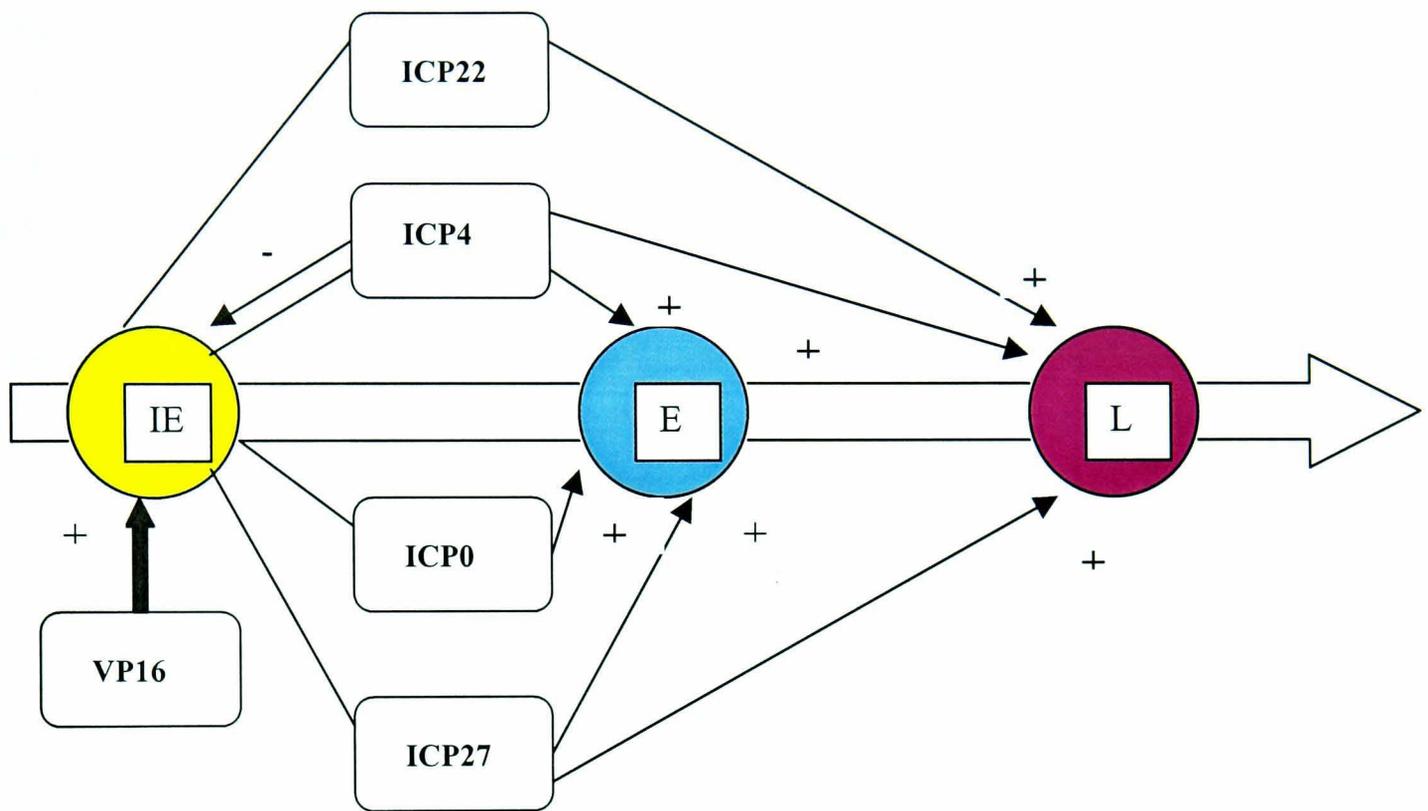


Figure 1.2 HSV-1 Gene Regulation

The immediate early (IE) genes are expressed immediately after infection in the absence of *de novo* protein synthesis. The VP16 virus tegument protein interacts with the cellular factor Oct1 to regulate the expression of IE genes by positively binding to their promoters. The IE gene products ICP4, ICP27, and ICP0 are responsible for activating early (E) genes. After viral DNA replication, the ICP4, ICP22, and ICP27 IE polypeptides regulate expression of the late (L) genes. ICP4 can also inhibit the expression of IE genes, including its own expression, once transcription of the E class has been initiated.

perfect palindrome of 144bp, while oriS exhibits a 45bp palindrome (Weller *et al.*, 1985; Knopf *et al.*, 1986; Lockshon and Galloway, 1986). Neither copy of oriS is essential, as deletion of one or both copies of oriS has no effect on viral DNA replication in cultured cells (Igarashi *et al.*, 1993).

Seven HSV-1 genes are necessary and sufficient for DNA replication: UL5, UL8, UL9, UL29, UL30, UL42, and UL52 (Wu *et al.*, 1988; McGeoch *et al.*, 1988). These encode the following proteins: a heterodimeric DNA polymerase (UL30, UL42), single-strand DNA-binding protein (also known as ICP8) (UL29), a heterotrimeric primosome with 5'-3' DNA helicase and primase activity (UL5, UL52, UL8), and an origin-binding protein with 3'-5' DNA helicase activity (UL9). HSV-1 also encodes a set of enzymes involved in nucleotide metabolism that are not required for viral replication in cultured cells. These enzymes include a deoxyuridine triphosphate (UL50), a ribonucleotide reductase (UL39, UL40), a thymidine kinase (UL23), an alkaline endo-exonuclease (UL12), and a uracil-DNA glycolase (UL2) (reviewed by Boehmer and Lehman, 1997).

Following DNA synthesis, late genes are expressed which lead to assembly of capsids in the nucleus. Cleavage and packaging of concatemeric DNA replication intermediates into preformed capsids is a tightly coupled process in which DNA cleavage occurs once a capsid is filled with one genome equivalent. Cleavage introduces an asymmetric cut in the *a* sequence, producing an L terminus that contains 18bp of the DR1 repeat and a single 3'-nucleotide extension, and an S terminus that contains 1bp of DR1 and a single 3'-nucleotide extension. Circularization of these ends reconstitutes a complete DR1 sequence that is shared between the terminal *a* sequences of the L and S components (Mocarski and Roizman, 1982). The exact molecular mechanism of the cleavage-packaging reaction is unknown, however several biochemical activities that may be involved in the process have been identified. These include virus-encoded proteins that specifically recognise the *pac2* site, an element within the *a* sequence responsible for cleavage and packaging (Chou and Roizman, 1989); a virus-induced DNA endonuclease that introduces double-strand cuts in the *a* sequence (Wohlrab *et al.*, 1991; Dutch *et al.*,

1994) and is part of an activity that promotes *in vitro* recombination of repeated *a* sequences (Bruckner *et al.*, 1992). In addition, mutational studies have implicated several non-structural HSV-1 gene products (UL6, UL15, UL25, UL28, UL32, and UL33 proteins) as well as several capsid proteins (UL18, UL19, and UL26.5 proteins) (Addison *et al.*, 1990; Al-Kobaisi *et al.*, 1991; Tengelsen *et al.*, 1993; Desai *et al.*, 1993). Assembled capsids leave the nucleus and gain tegument and envelope as they bud through the nuclear membrane, however the exact process is not fully understood. Following maturation virions leave the cell by exocytosis (Rixon, 1993).

1.5 HSV latent infection

HSV has been classified in the subfamily alphaherpesvirinae on the basis of its ability to establish and maintain latent infections in neurons. Latency has been defined as the persistence of the virus in a host in a non-infectious form. During latency the viral genome takes the form of a circular episomal element formed due to joining of the termini (Aurelian *et al.*, 1990). The latent genome is associated with cellular histones thus having a nucleosomal structure similar to that of cellular chromatin (Deshmane and Fraser, 1989). In contrast to the lytic pathway, viral gene expression is almost completely repressed during latency. This restriction prevents lysis of neurons and is a major feature of latency (Cann, 1993). During latency, transcription only occurs from a single area of the genome and results in a family of RNA molecules referred to as latency-associated transcripts (LATs).

1.5.1 Latency-Associated Transcripts (LATs)

In both HSV-1 and HSV-2, a single region of the genome, located in the long terminal repeat, known as the latency-associated transcript or LAT has been shown to encode RNA (Stevens *et al.*, 1987). The minor LAT product is 8.3kbp. The most abundant (i.e. major) LATs are 2 co-linear, predominantly nuclear poly(A)- RNAs of 2kbp and 1.5kbp, which are believed to be spliced from the 8.3kbp poly(A)+ LAT (Devi-Rao *et al.*, 1991; Farrell *et al.*, 1991; Spivack *et al.*, 1991; Wagner *et al.*, 1988). The major LATs appear to share 5' and 3' termini differing only in the excision of a small intron with unusual use

of GC instead of the consensus GU at the 5' consensus site (Wagner *et al.*, 1988; Spivack *et al.*, 1991). LATs can be readily detected in latently infected neurons, by Northern blot analysis (Wagner *et al.*, 1988) or by oligonucleotide probe (Zwaagstra *et al.*, 1990). However, protein expression from LATs has not been detected during latency.

LAT overlaps the ICP0 and ICP34.5 genes in an antisense direction. Thus, it was postulated that the transcripts influenced and maintained latency by down regulating ICP0 through an antisense mechanism (Farrell *et al.*, 1991). However, viruses with deletions in the promoter or the transcribed region of LAT are able to establish and maintain latency and reactivate.

Many researchers have shown that LAT deletion mutants demonstrate a reduced capacity to reactivate and the kinetics of reactivation of these mutants is slower than wild-type, suggesting that LATs play no role in the establishment and maintenance of latency, but rather play a role in efficient reactivation (Steiner *et al.*, 1989; Hill *et al.*, 1990; Block *et al.*, 1990; Perng *et al.*, 1994).

Chen *et al.*, (1997) analysed the effects of a mutation in the LAT locus on viral gene expression in latently infected mouse trigeminal ganglia. The mutation removed the promoter, transcriptional start site, and 1,015 bp of transcribed sequences of LAT. This mutant had reduced levels of the major LATs, which resulted in an increase in the accumulation of transcripts from the IE gene encoding ICP4, and an accumulation of transcripts from the early gene encoding thymidine kinase. Chen *et al.* (1997) thus concluded that a viral function associated with the LAT locus is the repression of the accumulation of at least two productive cycle genes in latently infected mouse ganglia. Perng *et al.*, (1996) recently showed that the first 1.5 kb of LAT is sufficient to generate wild-type levels of spontaneous reactivation from LAT-ve mutants. This region does not overlap any portion of any known HSV-1 gene.

1.5.2 Latency promoters

Two LAT promoters have been identified, LAP1 and LAP2. LAP1 is situated at the 5' end of the 8.3 kb LAT and is 700 bp upstream of the 2 kbp intron. Sequence analysis has demonstrated the presence of several *cis*-acting elements. Upstream elements specifically contribute to LAP1 function and sequences 620 nucleotides upstream of the transcription start site have been found to be responsible for full promoter activity (Soares *et al.*, 1996). LAP1 contains a TATA box basal element (Soares *et al.*, 1996) and proximal elements such as CAAT, USF₁, and Sp1, YY1, AP-2, and a potential binding site for the HSV-1 immediate early transactivator, ICP4 (Zwaagstra *et al.*, 1989). ICP4 negatively regulates expression directed from LAT constructs in cotransfection assays. (Batchelor and O'Hare, 1992).

A second promoter which lies between LAP1 and the 5' end of the 2 kb LAT has been identified and designated LAP2 (Soares *et al.*, 1996). It is considerably weaker than LAP1 (Goins *et al.*, 1994). It lacks a TATA box, yet contains elements found in a variety of housekeeping promoters such as a GC-rich and a C/T rich sequence separated by a stretch of 23 thymidine residues, all of which contribute to LAP2 activity *in vitro* (Goins *et al.*, 1997). The C/T rich element forms a non-B-DNA triplex structure and can be bound by a family of transcription factors (Bossone *et al.*, 1992; Pyrc *et al.*, 1992) as well as Sp1 (Goins *et al.*, 1997). A minor groove binding factor HMG I(Y) binds to the polyT stretch and thus facilitates the binding of Sp1 to LAP2 (French *et al.*, 1996). A region of LAT required for efficient reactivation has been mapped to LAP2 (Bloom *et al.*, 1996; Perng *et al.*, 1996), suggesting that this promoter may play a role in driving expression of some downstream gene important to the reactivation phase.

1.5.3 Reactivation

Reactivation of HSV results in anterograde axonal transport to susceptible cells of the dermis, resulting in recurrent productive virus infection at or near the initial site of infection. Lytic infection proceeds until the host immune response clears it. HSV can be induced to reactivate by a variety of stimuli. These include axotomy, elevations in cyclic AMP (cAMP), UV light, hyperthermia, or possible hormonal fluctuations e.g.

menstruation. The molecular basis of how HSV-1 reactivates from latency is unknown. It is clear that mutations that result in reduced viral replication efficiency in all cell types have a negative impact on both the establishment of latency and the ability to reactivate (Leib *et al.*, 1989; Katz *et al.*, 1990; Cai *et al.*, 1993). Mutations that result in replication deficits in nondividing cells such as thymidine kinase (TK)-negative mutants, also result in reactivation defects (Izumi and Stevens, 1990; Tenser, 1991). Mutations within the 5' end or promoter region of the latency-associated transcript (LAT) gene do not affect viral replication in any cell type but result in reduced reactivation *in vivo* in rabbits and mice (Bloom *et al.*, 1996; Hill *et al.*, 1996; Thompson and Sawtell, 1997). In the murine model, it has been demonstrated that LAT mutants establish significantly fewer latent infections, and this most likely accounts for the reduction in reactivation observed (Thompson and Sawtell, 1997). IE expression has been implicated in the switch from latency to the lytic phase. This is supported by findings that an increase in intracellular cAMP levels stimulates transcription of genes via a cAMP response element in the IE gene promoter (Wheatley *et al.*, 1992). Reactivation is thought to occur in the absence of any preexisting viral proteins. Because the immediate early protein ICP0 is the only HSV-1 protein expressed at very early times during productive infection and is capable of activating expression of all classes of viral genes (IE, E and L) (Cai and Schaffer, 1992), it has been suggested that low-level expression of ICP0 in neurons may be responsible for the initiation of productive-phase gene expression during reactivation (Leib *et al.*, 1989; Clements and Stow, 1989). Other viral gene products reported to facilitate reactivation include thymidine kinase and ribonucleotide reductase (Aurelian *et al.*, 1990).

1.6 HSV-1 Adsorption and Penetration

HSV-1 is an enveloped virus. For these viruses, entry into a cell requires binding of virus to receptors on the cell surface followed by endocytosis of the virion or by direct fusion of the virion envelope with the cell plasma membrane (Marsh and Helenius, 1989).

For enveloped viruses e.g. HSV, that penetrate by fusion with the plasma membrane, molecular interactions between virion surface components and cell surface components may be necessary, not only to permit binding of the virus to the cell but also to trigger fusion of the viral and cell membranes. The current hypothesis is that virus attachment is

a two-step process (McClain and Fuller, 1994) involving different glycoproteins and several receptors. gB and gC have been shown to be involved in the initial attachment phase, through interactions of positively charged glycoprotein structures with negatively charged heparan sulphate (HS) moieties located on cell surface proteoglycans (Lycke *et al.*, 1991; Sheih *et al.*, 1992). This HS-dependent attachment may facilitate a second attachment in which gD binds to a cellular receptor. Following attachment, the virus penetrates the cell by fusion of the virus envelope with the cell plasma membrane. This step involves several glycoproteins including gB, gD, gH, gL and gK (Spear, 1993). Endocytosis of HSV-1 does occur. This may be largely a dead-end route that leads to virus destruction in lysosomes (Campadelli-Fiume *et al.*, 1988(a); Wittels and Spear, 1991).

Following binding HSV-1 fuses with the plasma membrane. The envelope and many of the tegument proteins are lost, however, some remain associated with the capsid (Morrison *et al.*, 1998; Sodeik *et al.*, 1997). Next the capsid is transported through the cytosol to the nucleus where it binds to nuclear pore complexes (NPCs) (McClain and Fuller, 1994). Transport occurs along microtubules. Electron microscope pictures of the infection process show that the DNA is rapidly and efficiently ejected from the NPC-bound capsid, leaving behind an empty capsid that is eventually released into the cytosol (Sodeik *et al.*, 1997; Tognon *et al.*, 1981). Inside the nucleus, the incoming viral DNA localizes adjacent to the nuclear domain ND10 (Maul *et al.*, 1996) and results in its disruption (Maul *et al.*, 1993).

1.7 HSV-1 Egress

Herpesvirus nucleocapsids assemble in the nuclei of infected cells and acquire an envelope by budding through the inner nuclear membrane (Griffiths and Rottier, 1992; Roizman and Sears, 1996), however, the subsequent route of virus maturation and egress is uncertain. Two models have been proposed: the luminal model and the reenvelopment model.

Luminal Model: In this model capsids acquire envelope only from the inner leaflet of the inner nuclear membrane, and exit the cell by means of the host secretory pathway, remaining within luminal spaces throughout the entire trip. This model is based largely on the findings of Johnson and Spear (1982).

Reenvelopment Model: This model originally proposed by Stackpole (1969), involves the release of naked nucleocapsids into the cytoplasm by fusion of “primary enveloped virions” with the outer nuclear membrane. Final envelopment then occurs by budding into a cytoplasmic compartment before release from the cell via the host secretory pathway.

The controversy over which model is correct centres upon naked nucleocapsids that are frequently observed in the cytoplasm of infected cells. The luminal model states that these are aberrant fusion events and represent dead-ends, while the reenvelopment model recognises these as fundamental intermediates in the egress pathway. Neither model has been conclusively proved, however the weight of evidence favours the reenvelopment model. These include electron-microscopic studies which have been interpreted as showing final envelopment by budding into a late Golgi compartment or into cytoplasmic vesicles (Gershon *et al.*, 1994; Whealy *et al.*, 1991). Also, the phospholipid composition of secreted virions most closely resembles that of Golgi membranes (van Genderen *et al.*, 1994). If this two-stage envelopment model is correct, envelope proteins accumulate in the Golgi compartment or in Golgi-derived vesicles where final envelopment occurs.

Following egress, mature virions are capable of infecting neighbouring cells, however, it has been shown that the entry of HSV-1 from the extracellular medium and entry of the virus by cell-to-cell spread between adjacent cells apparently do not occur by identical processes (Roller and Herold, 1997).

1.8 Adsorption

1.8.1 Glycoproteins involved

To date in HSV, twelve membrane glycoproteins have been identified i.e. gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, gN - five of these (gB, gD, gH, gK, gL) have been shown to be important for viral infectivity. Several of these glycoproteins have been shown to play a major role in adsorption of virus to cell surface components.

The initial interaction of the virion with the cell is the binding of gC to heparan sulphate (HS) moieties of cell surface proteoglycans. The major evidence for this is as follows:

- Loss or alteration of cell HS significantly reduces the binding to cells (WuDunn and Spear, 1989)
- Heparin inhibits the binding of virus to cells (WuDunn and Spear, 1989), also virions can bind to heparin-affinity columns in physiological saline (WuDunn and Spear, 1989)
- HSV-1 gC null mutants are reduced in their ability to bind to cells with respect to wild-type virus (Herold *et al.*, 1991)
- Neutralising antibodies specific for HSV-1 gC can block the binding of virus to cells (Fuller and Spear, 1985; Svennerholm *et al.*, 1991)

Other alphaherpesviruses also initially bind HS by a alphaherpesvirus gC family member. When these gC family members were compared, it was seen that they all share a cluster of basic amino acids in the vicinity of a very hydrophilic region near the N-terminus. These sequences are good candidates for heparin-binding domains (Jackson *et al.*, 1991).

In HSV-1 another glycoprotein has HS-binding ability. gB has heparin-binding activity independent of gC (Herold *et al.*, 1991). For some cell types, absence of gB from the virion has little effect on virus binding provided gC is present (Herold *et al.*, 1991), but binding is dependent on gB when gC is absent.

1.8.2 Proteoglycans

A glycosaminoglycan (GAG) is a linear heteropolysaccharide possessing a characteristic disaccharide repeat sequence. One monosaccharide of the disaccharide repeat is an amino acid with D-glucosamine or galactosamine, and the other unit is typically, but not always, a uronic acid residue of either D-glucuronic acid or iduronic acid. Both units are invariably *N*- and *O*-sulphated, which adds to the heterogeneity of these complex macromolecules. Typically GAG chains are covalently attached at their reducing end through an *O*-glycosidic linkage to a serine residue or *N*-linked to asparagine in a core protein; the resulting macromolecule is termed a proteoglycan. Three of the most abundant GAGs on plasma membrane proteoglycans are heparan sulphate (HS), chondroitin sulphate (CS), and dermatan sulphate (DS) (Kjellén and Lindahl, 1991).

Proteoglycans are ubiquitous molecules present as integral membrane proteins of cells and as components of the extracellular matrix. Proteoglycans and their attached GAGs have a variety of roles in cell-cell and cell-matrix interactions and serve as activators of a variety of factors e.g. growth factors (Jackson *et al.*, 1991; Kjellén and Lindahl, 1991). A major function of cell surface proteoglycans is in cell adhesion and migration, dynamic processes that are mediated through interactions between the proteoglycan GAG chains and extracellular matrix (ECM), such as laminin, collagen and fibronectin. Proteoglycans also occur as integral components of basement membranes in probably all mammalian tissues. Interactions of these macromolecules with other ECM constituents contribute to the general architecture and permeability properties of the basement membrane, and thus these GAGs play a structural role (Jackson *et al.*, 1991).

1.8.3 Heparan sulphate glycosaminoglycans

Heparan sulphate proteoglycans serve as important regulators of cellular signalling by modulating, for example, the stability and biological activity of heparin-binding growth factors (Jackson *et al.*, 1991). HS chains are heterogenous, varying in sites of *N* acetylation, *N* sulphation, *O* sulphation, and epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA). Thus different subpopulations of HS might be present in different distributions on various cells types. Thus, it is plausible that this heterogeneity could

specify distinct receptors for various ligands, including HSV-1 and HSV-2. For example, heparin binds antithrombin III via a specific pentasaccharide structure which occurs infrequently within heparin chains near the nonreducing ends (Lindahl *et al.*, 1984). HS is distinguished from the closely related GAG heparin by its lower degree of sulphation, higher degree of N acetylation compared with the N sulphation of glucosamine residues, and the predominance of glucuronic acid rather than iduronic acid (Lindahl and Kjellén, 1991).

1.9 Penetration

It is proposed that multiple interactions involving virion glycoproteins and cell surface components are required to trigger fusion of the virion envelope with the cell plasma membrane, following initial binding to the cell surface.

gB, gD, and gH are required for penetration, but not for the initial binding of the virus to the cell surface (Cai *et al.*, 1988; Ligas and Johnson, 1988; Forrester *et al.*, 1992). Other glycoproteins thought to be involved include gK and gL. gK plays some role in controlling virus-induced cell fusion (Debroy *et al.*, 1985; Pogue-Geile and Spear, 1987).

gL forms a heterodimer (or higher oligomer) with gH - an association that appears to be essential for the proper processing and intracellular transport of both proteins (Hutchinson *et al.*, 1992).

Many cell surface receptor-ligand interactions trigger tyrosine phosphorylation and activation of signal transduction pathways. For example, the activities of fibroblast growth factors (FGFs) are mediated by a family of tyrosine kinase transmembrane receptors, the FGF receptors (Jaye *et al.*, 1992). Tyrosine phosphorylation may also play a role in cytomegalovirus (CMV) and human immunodeficiency virus entry (Briand *et al.*, 1997; Keay and Baldwin, 1996). Qie *et al.*, (1999) have shown that upon exposure to HSV-1 and HSV-2, at least three Vero cytoplasmic host cell proteins, p80, p104, and p140, become tyrosine phosphorylated within 5-10 minutes after viral exposure. No

phosphorylation is detected when the cells are exposed to a gL-deleted mutant, which binds, but fails to penetrate, suggesting that more than a physical binding of viral particles to cell surface receptors is required. Also from time studies, phosphorylation is associated with fusion of the viral envelope with the cellular plasma membrane. Therefore, tyrosine phosphorylation is triggered by viral entry.

The precise roles of gB, gD, and gH in viral penetration are as yet unknown. Interactions of these proteins with cell surface components somehow lead to fusion between the virion envelope and the cell plasma membrane.

1.10 Glycoprotein C (gC)

gC is a type I integral membrane protein (type I IMP), also known as group A IMPs (Garoff, 1985). A type I IMP contains an amino-terminal signal sequence that is cleaved during processing, a large external domain, a single transmembrane domain, and a hydrophilic cytoplasmic domain at its carboxy terminus. gC of HSV-1 is acquired by virions during budding of the nucleocapsid through the inner nuclear membrane (Griffiths and Rottier, 1992; Roizman and Sears, 1996).

1.10.1 Oligomeric structure

Glycoprotein C is the gene product of gene UL44 (Frink *et al.*, 1983; Draper *et al.*, 1984). The gene is non-essential for virus replication in cell culture, as shown by the isolation of virus mutants that fail to produce gC (Heine *et al.*, 1974; Holland *et al.*, 1983; Holland *et al.*, 1984). It encodes for a protein of *Mr* 54,995. It contains 511 amino acids, of which the first 25 constitute a cleavable signal sequence (Frink *et al.*, 1983; Kikuchi *et al.*, 1984). The protein has nine consensus sites for N-linked oligosaccharides (Frink *et al.*, 1983) as well as numerous O-linked oligosaccharides (Johnson and Spear, 1983; Olofsson *et al.*, 1983; Dall'Olio *et al.*, 1985).

gC contains eight cysteine residues which form 4 disulphide bonds (Rux *et al.*, 1996). The disulphide bond arrangement is Cys-1 (aa 127) to Cys-2 (aa 144); Cys-3 (aa 286) to

Cys-4 (aa 347); Cys-5 (aa 386) to Cys-8 (aa 442); Cys-6 (aa 390) to Cys-7 (aa 419). These domains have been shown to play key roles in gC e.g. the disulphide bond between Cys-1 and Cys-2 produces a loop which forms a binding region to the C3b fragment of the third component of human complement.

No function has been associated with the region of gC containing Cys-5 through Cys-8. However the disulphide bonds result in an extended loop which may stabilise the carboxy terminus. It is also thought to be important in maintaining the native, functional conformation of the protein (Rux *et al.*, 1996).

The transmembrane domain of wild type gC consists of a sequence of 23 hydrophobic amino acids, followed by a highly charged cytoplasmic domain consisting of 11 amino acids. The cytoplasmic domain of HSV-1 gC is essential for the stable anchoring of the glycoprotein in the cellular plasma membrane (Holland *et al.*, 1988). Although important for membrane anchoring, the domain appears to be dispensable for incorporation of the glycoprotein into virions (Holland *et al.*, 1988).

Unlike most other enveloped viruses, HSV virions display a distinctly disorganised arrangement of glycoproteins. Stannard *et al.*, (1987) showed by immunoelectron microscopy that gC appeared to consist of “randomly distributed” long, thin components which appear to extend approximately 20nm from the envelope. Handler *et al.*, (1996) calculated the relative proportions of each glycoprotein in a HSV virion. They calculated that there are approximately 4,900 molecules of gC per particle of HSV-1 (KOS).

1.10.2 HS binding domains

Glycoprotein C has been shown to be the principal glycoprotein involved in binding of HS moieties (Fuller and Spear, 1985; Herold *et al.*, 1991; Svennerholm *et al.*, 1991). Trybala *et al.*, (1994) reported the discovery of the sites of gC involved in HS binding. They elucidated this using a panel of anti-gC monoclonal antibodies, *mar* viruses carrying specific mutations in the gC gene, and synthetic peptides to localise the functional binding site.

The amino acid residues critical for gC-mediated attachment of HSV-1 to HS were localised to two separate regions of the glycoprotein: one including Arg-143, -145, -147 and Thr-150, and the other containing Gly-247 (Trybala *et al.*, 1994). While the two regions are separated by almost 100 amino acids, in the three dimensional structure they are in close proximity. This area is an Arg-rich sequence and of a polycationic nature. Other polycationic substances e.g. neomycin, poly-L-lysine and poly-arginine are known to compete with HSV attachment to cells (Langeland *et al.*, 1987, 1988, 1990; WuDunn and Spear, 1989; Campadelli-Fiume *et al.*, 1990). Thus similarly, it is suggested that the gC-HS interaction relies a great deal on ionic interaction.

1.10.3 gC null mutant viruses

Herold *et al.*, 1991 constructed a gC-negative virus, to determine the role of gC in adsorption and infectivity. Using HEp-2 cells, they found that significantly fewer gC-negative virions than either wild type or gB-negative virions bound to the cells. However binding of gC null mutants was more avid than wild type to cells, as they could not be removed by simple washing. This could indicate that binding of gB (the other HS binding glycoprotein) to HS or to its receptor is stronger than gC to HS. Also, there was a significant delay in the penetration of adsorbed gC-negative virus compared to wild type, but the rates of penetration for the two viruses were then comparable (Herold *et al.*, 1991).

For HEp-2 cells, when gC is present in the virion, the binding activity of gC predominates, and gB appears not to contribute significantly to initial viral attachment. For gC-negative viruses, gB plays the key role in the attachment of viruses (Herold *et al.*, 1991). Thus gB may interact when gC is missing or when the structural elements of HS to which gC binds are inaccessible or limited. These features may be present in different concentrations in different cell lines. Sears *et al.*, 1991, showed that wild type and gC-negative strains differentially infect the apical and basolateral surfaces of polarized MDCK canine kidney cells. The gC-negative strain could only infect cells from the

basolateral surface, whereas the wild type could infect from both surfaces. This could be due to the fact that in gC-negative virus the structures to which gB binds, are only present on the basolateral surface, whereas those with which gC interacts are present on both surfaces.

1.11 Glycoprotein B (gB)

Glycoprotein B is the gene product of gene UL27. gB is among the most highly conserved herpesvirus structural components, suggesting a common and essential role in the life cycle of the *Herpesviridae* (Albrecht and Fleckenstein, 1991; Pellet *et al.*, 1985b). gB is expressed as an early gene product that persists following viral DNA synthesis, indicating that it is a member of the $\gamma 1$ temporal class of genes. The gB structural gene encodes 904 amino acids (Bzik *et al.*, 1984). Biochemical analysis of gB has demonstrated that it contains a 30-residue N-terminal signal sequence that is cleaved during processing, a 967-residue external domain, a 68-residue transmembrane domain that is predicted to span the membrane three times, and a 109-residue cytoplasmic domain (Bzik *et al.*, 1984; Cai *et al.*, 1988; Claesson-Welsh and Spear, 1987; Pellett *et al.*, 1985b). The molecule forms minimally a homodimer during or soon after the process of translation and is subsequently glycosylated through a series of successive stages in the rough endoplasmic reticulum involving carbohydrate addition to six consensus sites for N-linked glycosylation (Chapsal and Pereira, 1988; Claesson- Welsh and Spear, 1986; Kousoulas *et al.*, 1983). The functional form of gB is an oligomer (Cai *et al.*, 1988) and it was seen that oligomer formation required a 28- amino acid movable domain consisting of residues 626 to 653 (Laquerre *et al.*, 1996). Within this domain it was shown that a Cys residue at position 633 is essential for folding, processing, and incorporation into mature infectious virus particles. gB is further processed in the Golgi complex and is transported to the surface of infected cells. The pathway for gB insertion into the viral envelope is thought to involve diffusion or active transport of immature gB to the inner nuclear membrane where it is initially acquired, along with the other envelope glycoproteins during the process of virion budding (Darlington and Moss, 1968, Gilbert and Ghosh, 1993; Gilbert *et al.*, 1994, Torrisi *et al.*, 1992)

By immunoelectron microscopy, Stannard *et al.*, (1987) showed that gB appeared in the HSV virion as clustered “T-shaped” spikes. These form the most prominent spikes, extending about 14nm from the envelope. Unlike gC, gB was in patches which were distinct from one another. From Handler *et al.*, (1996), the quantity of gB in the virion envelope was similar to gC in both HSV KOS and HSV NS (i.e. approx. 4,900 molecules).

1.11.1 Role in Adsorption and Penetration

Although gB is able to mediate virus binding, at least when gC is absent, it is unknown whether this binding is distinct from the normal pathway, only occurring when gC is absent, or whether it occurs alongside gC-binding, though is only responsible for a small proportion of viral glycoprotein binding. gB is also required for viral penetration and for HSV-induced cell fusion (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Cai *et al.*, 1988), whereas gC is dispensable for these two processes (Manservigi *et al.*, 1977; Homa *et al.*, 1986). Therefore, the possibility exists that the interaction of gB with HS is important in part for its contribution toward activation of membrane fusion.

1.11.2 HS binding domains

gB contains a lysine-rich (pK) sequence (amino acids 68-76: KPKNKKPK). This sequence is similar to the consensus sequence predicted for the HS binding domain of proteins (Cardin and Weintraub, 1989; Trybala *et al.*, 1994). Laquerre *et al.*, (1998c) deleted this sequence from gB to determine what role this played in HS binding, viral entry and cell-to-cell spread. They found that the pK sequence is not essential for gB processing or function in infection; is responsible for HS binding; and the mutant gB molecule lacking the pK sequence proved functional for virus penetration by rescue of gB null mutant virus.

1.11.3 gD and its receptors

Glycoprotein D is the gene product of gene US6 (Watson *et al.*, 1982). This gene is essential. It encodes for a protein of Mr 43,344, and contains 394 amino acids. It functions in adsorption, binding to several cellular ligands. It contains a cleavable N-

terminal signal sequence. It has a hydrophobic sequence near its N-terminus, a hydrophilic sequence near its C-terminus which functions as a membrane-spanning domain and several sites for N-linked and O-linked glycans (Campadelli-Fiume and Serafini-Cessi, 1985). At the amino acid level, gD from HSV-1 (gD-1) is 85% identical to its homologue in HSV-2 (gD-2) (Lasky and Dowbenko, 1984; Watson, 1983; Watson *et al.*, 1982). The two proteins are functionally interchangeable (Muggeridge *et al.*, 1990b, Ruyechan *et al.*, 1979) and give rise to type-common and type-specific monoclonal antibodies (Muggeridge *et al.*, 1990a). gD-1 and gD-2 have high structural and functional homology (Chiang *et al.*, 1994; Lasky and Dowbenko, 1984; Muggeridge *et al.*, 1990b; Ruyechan *et al.*, 1979; Watson, 1983). Both proteins have identical disulphide bond patterns (Long *et al.*, 1992) and analogous mutations in gD-1 or gD-2 have similar effects on antigenicity and function of both proteins (Chiang *et al.*, 1994).

1.12 Other HSV binding ligands

Human and animal representatives of the alphaherpesvirus subfamily exhibit common requirements for entry into cells (Mettenleiter, 1995; Spear, 1993). A cloning strategy has been devised for isolating plasmids encoding cell surface proteins that can mediate herpes simplex virus type 1 entry (Montgomery *et al.*, 1996).

This assay relies on the use of Chinese hamster ovary (CHO) cells, which express GAGs required for virus binding to cells but are resistant to the entry of certain HSV-1 strains such as HSV-1(KOS) (Sheih *et al.*, 1992). Expression plasmids or subdivisions of the libraries, that contain plasmids capable of conferring susceptibility to HSV-1(KOS) can be identified by transfecting the CHO cells and then assaying for infectivity with a recombinant HSV-1(KOS) expressing a reporter gene. This method was used to isolate a previously undescribed member of the human TNF receptor family, which was originally called herpesvirus entry mediator (HVEM), now termed HveA (herpesvirus entry protein A) (Montgomery *et al.*, 1996). HveA is a type I membrane glycoprotein with cysteine-rich repeats in the ectodomain that are characteristic of the TNF receptor family (Montgomery *et al.*, 1996) and with a cytoplasmic domain that can interact with members of the TRAF family of signalling molecules (Hsu *et al.*, 1997; Marsters *et al.*, 1997).

HveA also binds to isolated HSV-1 or HSV-2 gD and to gD in virions (Nicola *et al.*, 1998; Whitbeck *et al.*, 1997). The stage of HSV entry at which HVEM operates must be either the membrane fusion reaction that occurs after binding of virus to cell-surface GAGs, or the release of internal virion proteins, including VP16, from sites of entry and their transport to the cell nucleus i.e. virus binding to CHO-K1 cells occurs efficiently in the absence of HVEM or in the absence of anti-HVEM antibody (Montgomery *et al.*, 1996). By use of anti-HveA antibodies that blocked HSV entry, it was shown that HveA serves as the principal co-receptor for entry of HSV-1(KOS) into activated human T lymphocytes (Montgomery *et al.*, 1996). The antibodies did not protect a number of other human cell types from infection, however, indicating that there must be other co-receptors for HSV entry. Another indication for the existence of multiple independent receptors for HSV entry was the finding that, although HveA expression in CHO cells enhanced the entry of all wild type HSV-1 and HSV-2 strains tested, they failed to mediate the entry of three mutant HSV-1 strains (Montgomery *et al.*, 1996) - HSV-1(KOS)Rid1, HSV-1(KOS)Rid2, and HSV-1(ANG). The gD from these mutant viruses also failed to bind HveA, whereas wild-type forms of HSV-1 and HSV-2 gD were able to bind (Whitbeck *et al.*, 1997)

A second mediator of HSV entry was shown to be poliovirus receptor-related protein 2 (Prr2) (Eberle *et al.*, 1995). No function and no poliovirus receptor activity have been reported for this protein, and it was therefore designated herpesvirus entry mediator B (HveB) (Warner *et al.*, 1998) or Nectin2 (Takahashi *et al.*, 1999). HveB mediates the entry of HSV-2 strains, PRV, and certain mutants of HSV-1, (KOS)Rid1, (KOS)Rid2, ANG), but fails to mediate the entry of wild-type HSV-1 strains or BHV-1 (Warner *et al.*, 1998). This data shows that multiple alphaherpesvirus receptors exist, and differ in their specificities for individual viruses in the subfamily.

Because HveB is closely related to the poliovirus receptor (Pvr) (Mendelsohn *et al.*, 1989) and to the poliovirus receptor-related protein 1 (Prr1) (Lopez *et al.*, 1995), the possibility that one or both of these proteins might mediate the entry of HSV-1 and -2 as well as PRV and BHV-1 was explored. Prr1 mediated the entry of several HSV-1 strains

and three HSV-1 mutants (ANG, Rid1, Rid2) with amino acid substitutions in gD that preclude the use of HveA for entry (Montgomery *et al.*, 1996). Prr1 designated as HveC (Geraghty *et al.*, 1998) or Nectin1 (Takahashi *et al.*, 1999), also enhanced infection by HSV-2 strains (Sheih *et al.*, 1992). HveC expression rendered CHO cells susceptible to PRV and BHV-1 as well as HSV, entry being a function of virus dose. The region of gD-binding activity and that functions in HSV entry is located in the V domain of its ectodomain (Cocchi *et al.*, 1998; Krummenacher *et al.*, 1999). Pvr mediated the entry of PRV and BHV-1 but not of the HSV-1 strains. The fact that HveC and Pvr (designated Pvr-HveD), both of which are human molecules, can mediate entry of PRV and BHV-1 does not imply that human cells could support the replication of those viruses; it does suggest, however, that the animal homologues of HveC and Pvr-HveD could mediate entry of those viruses into cells of the natural hosts. Expression of HveA is detected principally in lymphoid organs (Kwon *et al.*, 1997; Hsu *et al.*, 1997), whereas HveB, HveC, and Pvr-HveD can be expressed in cells of the nervous system (Warner *et al.*, 1998; Mendelsohn *et al.*, 1989) or in cells cultured from the nervous system. The cellular role of HveB and HveC is that of intercellular adhesion molecules. They are anchored to the actin cytoskeleton and are recruited to cadherin-based adherens junctions through binding to L-afadin (Takahashi *et al.*, 1999; Lopez *et al.*, 1998).

HveB and HveC can be expressed individually or together in various human cell types of neuronal, fibroblastic, and epithelial origin (Warner *et al.*, 1998). In these cell types, either or both of these proteins could be important entry mediators for HSV-2 strains, and rare variant HSV-1 strains, whereas the wild-type HSV-1 strains would be able to use HveC, but not HveB. Thus it is possible that the variable ability of different HSV strains to utilise HveA, HveB, and HveC (and other unidentified proteins) for entry and variable expression of these entry proteins in different cell types will govern in part any strain-specific and serotype-specific patterns of viral spread in infected persons.

Cocchi *et al.* (2000) report HveC as a cellular function involved in cell-to-cell transmission of HSV-1, both in cells expressing HveC cDNA as a transgene and in a variety of human cell lines. Cell-to-cell spread, like entry, involved the V domain. Taken

with its expression in numerous human tissue types, this makes HveC a likely candidate as a mediator of virus spread to tissues that are the target of HSV infection in humans. Cocchi *et al.* (2000) propose that it promotes cell-to-cell spread by establishing the necessary intercellular contacts between juxtaposed cells, both by engaging in homophilic interaction and by interaction with viral gD.

A further gD receptor, 3-O-sulphated heparan sulphate, has recently been found by Shukla *et al.*, (1999). By screening CHO cells resistant to HSV entry transfected with cDNA, isoforms of D-glucosaminyl 3-O-sulfotransferases (3-OSTs) were shown to confer susceptibility to HSV entry (3-OST-3_A and 3-OST-3_B). Members of the family 3-OSTs are responsible for the addition of sulfate groups at the 3-OH position of glucosamine in HS. They modify HS late in its biosynthesis and each isoform recognizes as substrate, glucosamine residues in regions of the HS chain having specific, but different, prior modifications, including epimerisation and sulfation at other positions (Liu *et al.*, 1999). Thus different 3-OSTs generate different potential protein-binding sites in HS. From Shukla *et al.*, (1999) the enzyme-modified HS, rather than the enzymes themselves served as receptors to permit HSV-1 entry, as heparinase blocked entry of HSV-1 to cells. 3-OSTs are expressed in a variety of human tissues and cells (Shworak *et al.*, 1999) and may help explain the broad host range of HSV-1.

1.13 HS binding domain-deleted virus

Laquerre *et al.*, 1998(c) constructed a virus which was gC-negative and gB pK-negative, thus all its HS binding domains were deleted i.e. termed KgBpK⁻gC⁻. The rate of cell bound KgBpK⁻ (HS binding domain of gB deleted) mutant virus penetration was reduced compared to wild type virus, indicating that gB recognition of HS was required for this process. A gC null virus (KCZ) also entered cells more slowly, whereas the double mutant (KgBpK⁻gC⁻) virus did not differ significantly from either single-mutant virus, suggesting that HS recognition by both glycoproteins is required for efficient virus entry and that they may cooperate in this process. Also the double-mutant entered cells with a kinetics similar to wild type virus on sog9 cells (a glycosaminoglycan-deficient L-cell line) (Laquerre *et al.*, 1998c). This suggests that deletion of the HS binding function of

gB does not alter the fusion function of gB. Also, since the double-mutant was capable of binding to the cell surface through a non-HS receptor, but demonstrated a lower rate of penetration than wild type virus, viral binding to the inherent non-HS receptor can substitute for HS binding in promoting virus entry, but with less efficiency.

Laquerre *et al.*, (1998c) also observed the ability of the mutant viruses to form plaques in the presence of virus-neutralising antiserum, where plaque formation is dependent on cell-to-cell spread. They showed that the KgBpK⁻ virus produced smaller plaques than wild type, while gC-negative virus produced plaques larger than wild type, and KgBpK⁻ gC⁻ virus produced plaques similar in size to wild type virus. Thus, gB binding to the HS receptor is required for efficient intercellular infection.

1.14 HSV entry into primary neurons

HSV in its normal life-cycle, mainly infects two distinct cell types, epithelial and neuronal cells (Roizman and Sears, 1993). Most of the work on HSV-1 has been carried out using epithelial cells. However, it is unknown whether the requirements for HSV-1 entry may vary between the two cell types, as has been postulated for other viruses e.g. human immunodeficiency virus type 1 (HIV-1) (Bhat *et al.*, 1991; Peudenier *et al.*, 1991; Parmantier *et al.*, 1995). It is possible that a different family of GAGs may mediate binding in neurons, or that they may be present in different concentrations on neuronal cells, or may possess different structural elements.

To investigate the entry requirements Immergluck *et al.*, 1998, used embryonic chick brain as a model, and compared entry with fibroblast cells. They showed that less soluble heparin was needed to inhibit entry to chick fibroblast cells than primary neurons. This may be because neuronal cell surface contains more HS receptors than fibroblasts, or that the virus may have a higher affinity for neuronal HS than fibroblast HS, or that soluble heparin may not resemble neuronal HS as well as fibroblast HS.

A gC-negative virus was used to infect the primary neurons, and it was seen that it had a reduced specific binding activity and specific infectivity compared to the parental wild

type virus or gC⁺ virus in fibroblasts (Immergluck *et al.*, 1998). There was no impairment in the ability of the gC-negative virus to bind to neuronal cells (virion particles bound per cell). However, there was a marked reduction in the ability of the gC-negative virus to infect neuronal cells and express an immediate early reporter gene, β -gal (Immergluck *et al.*, 1998).

These results obtained for neurons suggest that gC does not play the same role in initial binding to neurons as it does to fibroblast or epithelial cells, but it does play a key role in entry at a step beyond the initial binding of virus to the cell surface.

1.15 Cell tropism and serotype differences in viral entry

Although HSV-1 and HSV-2 bind HS, the serotypes exhibit differences in epidemiology, cell tropism, and susceptibility to inhibitors of viral binding that may reflect differences in viral entry. For example, HSV-1 is more likely to cause oral lesions and sporadic encephalitis, whereas HSV-2 commonly causes genital lesions. For HSV-1, but not HSV-2, gC plays the predominant role (at least *in vitro*) although gC-2 may contribute to the interactions of HSV-2 with cell surface HS (Gerber *et al.*, 1995). It seems likely that gB-2, the other heparin-binding glycoprotein, may play the key role in mediating HSV-2 binding. Thus, the differences in the relative contribution of gC and gB to viral binding for HSV-1 and HSV-2 may help to explain differences in tropism and viral pathogenesis. For example, cells that preferentially bind HSV-1 may express more gC-specific structural features of HS, whereas cells that preferentially bind HSV-2 express more gB-specific structural features of HS.

The heterogeneity of the various HS proteoglycans expressed on cell types could specify distinct receptors for various ligands, including HSV-1 and HSV-2. Binding of HSV-1 to Vero or HEp-2 cells is mediated principally by gC-1 (Herold *et al.*, 1994; Herold *et al.*, 1991; Svennerholm *et al.*, 1991; Tal-Singer *et al.*, 1995), with gC-deleted mutants showing decreased specific binding activities, marked lag in penetration and reduced specific infectivities (Herold *et al.*, 1991). In contrast, an HSV-2(G) mutant with gC-2 deleted shows no impairment in specific binding activity, rate of penetration, or specific

infectivity on Vero or HEp-2 cells (Gerber *et al.*, 1995). Thus, it suggests that gB-2, the other HS-binding glycoprotein, plays a key role in HSV-2 attachment to these cells. Most viruses bind at low temperatures (0 to 4°C) but penetrate cells only at physiological temperatures. For some viruses, a secondary binding step follows the initial binding at 4°C (Haywood, 1994). This secondary binding generally occurs only at temperatures permissive for penetration and may be required for penetration. Herold *et al.*, 1996 showed the differences in HSV-1 and HSV-2 binding at 4°C and 37°C by comparing the effects of modified heparin compounds during the adsorption period at these two temperatures. They have shown that the initial step in HSV-1 adsorption may be a low-affinity interaction between gC-1 and HS structures. This initial binding is observed at 4°C and promotes efficient adsorption, but is not necessary. This interaction may serve to concentrate virions at the cell surface, and at physiological temperatures, may promote a conformational change in the virus so that a second, higher-affinity interaction with HS occurs. The second interaction is mediated primarily by gB-1 binding either to a different subset of HS structures or with higher affinity to similar HS moieties (Herold *et al.*, 1995). Studies conducted at physiological temperatures primarily reflect this second interaction, as evidenced by the observation that there are no significant differences between wild-type viruses (containing both HS-binding glycoproteins, gB and gC) and viruses with gC-1 deleted (containing only gB) with respect to susceptibility to inhibitors of viral adsorption at 37°C; differences are only detected at 4°C. This second interaction may be essential and presumably triggers subsequent heparin-independent interactions between the virus and the cell surface that culminate in virus-cell fusion. Subsequent interactions probably include interactions between gD and/or gH-gL and other cell surface components (Fuller and Lee, 1992; Johnson *et al.*, 1990; Johnson and Ligas, 1988; Karger and Mettenleiter, 1993; McClain and Fuller, 1994). Minimal temperature-dependent differences were seen for HSV-2 i.e. binding at both temperatures may be mediated principally by only one glycoprotein, presumably gB-2. Thus, there may be differences in the structural sequences of HS with which HSV-1 and HSV-2 preferentially bind and differences in initial (evident at 4°C) and secondary (evident at 37°C) interactions with cell surface HS. These differences in the roles of gC and gB in viral binding, may contribute to the observed differences in epidemiology and cell

tropism. Also, Shukla *et al.*, (1999) have shown that the action of D-glucosaminyl 3-O-sulfotransferases (3-OSTs) creates modified HS which binds to gD. Cells expressing 3-OSTs are more susceptible to HSV-1 than to HSV-2, thus this could also play a role in determining cell tropism.

1.16 Motor neuron infection by HSV-1

HSV-1 infections begin at sites of the host exposed to the environment e.g. the epithelial linings of the pharyngeal tract, the eyes. Virus replication usually occurs first in nonneuronal cells, followed by spread of virus into afferent (e.g. sensory) or efferent (e.g. motor) nerve fibres innervating the infected tissue. Typically HSV-1 establishes a latent infection predominantly in sensory nerves, however, infection of motor nerves occurs. Early studies by Sabin (1938) first demonstrated that the site of infection influenced transneuronal spread of virus. Motor neuron infection in experimental animals can be achieved by infection of the animal at several sites e.g. tongue, gastrocnemius muscle. Dobson *et al.*, (1990) showed that HSV-1 can establish a latent infection in motor neurons. Expression of a β -galactosidase marker within the virus in motor neurons was seen to be transient with respect to expression of the marker in sensory neurons. This was thought to be due to the choice of promoter (Maloney murine leukaemia virus long terminal repeat promoter) driving the marker (Dobson *et al.*, 1990). Yamamura *et al.*, (2000) injected rats with an attenuated HSV expressing β -galactosidase under the LAT promoter in the gastrocnemius muscle. The virus was introduced into the anterior horn motor neurons of the spinal cord where β -gal activity was observed without apparent tissue destruction or inflammation. The expression of β -gal activity was recognised in 90% of the motor neurons and lasted for over 182 days, with expression occurring during the latent phase of the virus.

1.17 ICP34.5

In 1983 Thompson *et al.*, identified a neurovirulence locus in the long repeat region of the HSV-1 genome. Subsequently the gene was identified and called $\gamma_134.5$ by Chou and Roizman (1986) in strain F, and RL1 in strain 17+ (Dolan *et al.*, 1992). The gene product

of this locus is called ICP34.5. Following intracranial inoculation, mutants in this region are 100,000 times less virulent than wild type viruses (Taha *et al.*, 1989; Chou *et al.*, 1990; MacLean *et al.*, 1991). The RL1 promoter is found partially within the α sequence and it lacks a canonical TATA box.

Two common strains of HSV-1, F and 17+ show homology in ICP34.5 with the exception of a peptide repeat PAT present 10 times in strain F but only 5 times in strain 17+ (Dolan *et al.*, 1992). Deletion mutants of RL1 include 1716, a 759 bp deletion, that is located within each copy of the *Bam*HI *s* fragment (0 to 0.02 and 0.81 to 0.83 map units). The deletion removes one complete copy of the 18bp DR1 element of the α sequence and terminates 1105 bp upstream of the 5' end of IE1. (MacLean *et al.*, 1991). Several other RL1 mutations have been made by introducing stop codons downstream of the initiating methionine.

At some sites of primary infection e.g. footpad, vagina, the replication of ICP34.5 mutants is much less than that of wild type virus, whereas in others e.g. eye, replication appears to be almost completely inhibited (Spivack *et al.*, 1995). ICP34.5 -mutants establish latency inefficiently and have been reported to reactivate far less efficiently than wild type virus as measured by explant *in vitro* reactivation from mouse ganglia (Robertson *et al.*, 1992; Perng *et al.*, 1995).

The function of the protein has shown to be cell type and cell state dependent (Brown *et al.*, 1994a). In mouse 3T6 cells, the lack of ICP34.5 results in a defect in maturation and the egress of virus from infected cells (Brown *et al.*, 1994b). In the human neuroblastoma SK-N-SH cell line, infection with ICP34.5-negative HSV results in preclusion of host cell protein synthesis via the protein kinase PKR pathway (Chou and Roizman, 1992, Chou *et al.*, 1995).

ICP34.5 is thought to function by complexing with proliferating cell nuclear antigen (PCNA), a protein involved in DNA replication and repair. This fact and their selective replication capacity has led to the development of ICP34.5 null mutants in the treatment

of brain tumours (Rampling *et al.*, 2000; Markert *et al.*, 2000). In tumour cells, PCNA levels are high and so ICP34.5 is not required for productive HSV replication, whereas in the surrounding nondividing, fully differentiated cells, PCNA levels are low, and so ICP34.5 is an absolute requirement for viral replication.

A 63-amino-acid carboxyl-terminal domain of ICP34.5 has been shown to have significant homologies (McGeogh and Barnett, 1991) with the carboxyl domains of the mouse myeloid differentiation protein MyD116 and the hamster growth arrest and DNA damage protein GADD34 (Fornace *et al.*, 1989). The amino-terminal portions of the proteins are quite diverse, and the sizes of the 3 proteins also vary (ICP34.5: 248 aa; MyD116: 657aa GADD34: 590aa). It has been shown that the carboxyl-terminal 63 amino acids are essential but not necessarily sufficient for the prevention of host cell shutoff by ICP34.5 and can be replaced by the homologous domain of MyD116 (He *et al.*, 1996).

The roles of MyD116 and GADD34 in the cell appears to be in blocking growth and DNA replication after damage, and the genes may act as tumour suppressor genes. However, the expression of MyD116 or GADD34 does not determine permissivity for ICP34.5-negative HSV (Brown *et al.*, 1997).

1.18 Viral vectors for gene therapy

One of the principal goals of gene therapy has been the development of a “magic bullet” – a vector that could be used to deliver therapeutic genes efficiently and accurately to any preselected tissue or cell type in the body. Ideally the vector would be administered by injection or infusion, whereupon it would accumulate and deliver the gene in the target organ or cells, but not into the surrounding neighbouring cells. Viruses are seen as ideal candidates for vectors e.g. retroviruses, adenoviruses, herpesviruses because of the natural viral mechanisms which efficiently and effectively deliver DNA to the host cell nucleus. At present no virus is ideally suitable for use as viral vector and several techniques are being used to improve their capabilities.

The first requisite of an injectable vector is that it should be preferentially retained in the organs and tissue that harbour the target cells. One strategy to ensure this is to modify the binding characteristics of these vector particles. One way has been to genetically modify the virus coat proteins e.g. retrovirus (Valsesia-Wittmann *et al.*, 1996), adenovirus (Watkins *et al.*, 1997), herpesvirus (Laquerre *et al.*, 1998a). An alternative strategy has been to use soluble bifunctional crosslinkers that bind both to the vector particle surface and to a cell-surface receptor, thereby providing a molecular bridge to anchor the vector particle to a targeted receptor (Wickham *et al.*, 1997). A combined approach is to display an immunoglobulin binding domain on the vector as a genetic fusion to the coat protein, and then use a monoclonal antibody to crosslink the vector with the targeted cell (Ohno *et al.*, 1997). A fourth approach has been to replace the coat proteins of the vector with the coat proteins of another virus that already has a desired host range. For example, retroviral vectors have been targeted to CD4-expressing cells by adding HIV gp120 to the retrovirus (Indraccola *et al.*, 1998). CD4 was also incorporated into the HSV genome (Dolter *et al.*, 1993), however, very low levels of the protein were incorporated into the viral envelope. Attempts to demonstrate that CD4 in the HSV envelope can interact with HIV-1 *env* on a cell surface to promote membrane fusion proved unsuccessful.

1.18.1 Viral vectors for CNS applications

Specific behavioural and pathological changes can result from the altered expression of small groups of genes or single genes within individual neuronal populations. Changes in the expression of specific genes in localized brain areas have been observed in association with particular diseases e.g. Alzheimer's disease (Mullan, 1992), amyotrophic lateral sclerosis (Rosen *et al.*, 1993). Current approaches using drugs have proved moderately successful, however often they cannot be directed towards a specific target, nor be specific or selective enough to be targeted directly to specific targets.

Viral vectors have been suggested as ideal candidates for use in the treatment of CNS diseases. The premise is based on the inoculation of a viral vector within the nervous system to produce localized expression of a gene of interest. This also has the advantage that several genes can be transmitted simultaneously. At present vectors for gene transfer

to the CNS have been developed from adeno-, adeno-associated, retro- and herpes viruses. All of these have many advantages and disadvantages.

- Adeno- and adeno-associated viral vectors

Adenoviruses are linear double stranded DNA viruses that are pathogenic in humans, producing respiratory and intestinal infections. Their major advantage is that they can infect a wide variety of cell types, including post-mitotic cells, and can be obtained at high titre (Barkats *et al.*, 1998; Kozarsky and Wilson, 1993). However the disadvantages of this system include high immunogenicity, toxicity at high titres and inability to sustain long-term expression of the transgene (Barkats *et al.*, 1998).

Adeno-associated virus (AAV) is a non-pathogenic member of the parvovirus group that requires coinfection with a helper virus (adenovirus or HSV) to replicate. They have several advantages for use as a viral vector: they can transfer foreign genes into neuronal cells, although at low efficiency; they are physically stable which allows purification, storage and *in vivo* administration; they have low toxicity; they have the ability to maintain long-term and stable transgene expression. However, the primary disadvantage of AAV-based vectors is that the insert size is limited to a maximum of approximately 5kb. Also efficient large-scale production of high-titre AAV is difficult to achieve (Snyder, 1999).

- Retroviral vectors

The most widely used vector systems in gene therapy are replication-defective murine and avian retroviruses packaged into infectious virions with helper virus. However, retroviral integration and gene expression requires target cells that are undergoing cell division (Miller *et al.*, 1990), and therefore neurons cannot be infected. Recently however, non-pathogenic retroviral vectors based on lentivirus have been developed (Olsen, 1998; Poeschla *et al.*, 1998). They can infect non-dividing cells in a long-lasting manner, delivering foreign genes in the absence of tissue pathology or immune reaction.

1.18.2 Herpes simplex virus vectors

HSV-1 has been suggested as a viral vector candidate for delivery to the nervous system. It has many advantages: it infects neurons efficiently; natural infection in humans

includes a latent state in which the viral genome persists in a non-integrated form in neurons, without causing disease in the immune-competent host. In this state it is transcriptionally active, and this latency-specific promoter system can be used to express transgenes (Fink *et al.*, 1996). HSV-1 is ideal for the delivery of multiple genes because of the large size of its genome (152kb) and the fact that approximately half of the gene functions may be deleted without blocking viral replication, allowing it to accommodate extensive foreign genetic sequences. A further advantage is the wide host range and so is not limited in its range of target cells.

Some concerns exist using HSV as a viral vector. Immune-mediated toxicity has been noted in experimental animals after direct injection of recombinant HSV-1 into the brain, especially when animals had generated a peripheral immune response to the vector (Wood *et al.*, 1994). 90% of humans are thought to have circulating antibodies to HSV-1 (Thomas *et al.*, 2000). This immune sensitisation can enhance autoimmune reactions to self or transgene antigens. Triggering of autoimmune diseases has been noted as a consequence of virus infections in “immune-privileged” organs such as the brain and eye (Levin *et al.*, 1998; Zhao *et al.*, 1998), due in part to antigen mimicry. Thus, vectors derived from a variety of viruses bearing or expressing antigenic proteins can potentially create damaging immune reactions in the short term (weeks to months) or long term (years).

A further safety issue is that the introduction/expression of virus proteins may activate or facilitate replication of endogenous pathogenic viruses, or that replication-competent recombinant virus may be generated. Analysis of human brains at autopsy suggest that almost half bear latent HSV-1 (Sanders *et al.*, 1996) and potential activation and replication of this virus in the brain could cause fatal encephalitis, with heightened risk to immune-compromised patients. Experimental studies to date (Sundaresan *et al.*, 2000) support the consensus that HSV vectors do not activate latent HSV-1, however, this is a relative parameter and may vary with different versions of HSV vectors and the biological status of individual subjects.

Three types of HSV-1 vectors are commonly in use: amplicons, replication-defective and replication-competent vectors.

- Amplicons: these are plasmid-derived vectors engineered to contain both an *Escherichia coli* and HSV origin of replication and HSV cleavage-packaging recognition sequences. Due to their production method, samples are contaminated with high percentages of unwanted helper virus particles. This problem has been circumvented by using packaging-defective cosmid- or HSV-1 bacterial artificial-chromosome-based transfection systems to provide the helper functions (Frafel *et al.*, 1996; Stavropoulos and Strathdee, 1998). The main advantages of these vectors are that they are easy to construct and multiple copies of the transgene (up to 15) can be delivered to neurons within each viral capsid; however the size of the transgene is limited to 10kb

- Replication-defective vectors: these are made of mutant viruses with deletions in one or more genes essential for the lytic cycle. Thus these need complementing cell lines for production. Several replication-deficient vectors have been constructed in which the immediate-early genes, whose expression produces ICP0, ICP4, ICP27 and ICP22 have been deleted or inactivated in various combinations (Marconi *et al.*, 1996; Wu *et al.*, 1996). Deletion of all IE genes prevents virus toxicity for cells at high multiplicity of infection, allowing the vector to persist for long periods (Samaniego *et al.*, 1998). However, elimination of ICP0 also reduces the level of transgene expression in cell culture. The advantages of these vectors are the low toxicity and the possibility of introducing multiple transgenes in the viral genome. However, their principal limitation is the inability to sustain long-term expression of the transgene in the brain. They are also difficult to grow to high titre and require cell lines expressing multiple complementing genes – these are also difficult to maintain.

- Replication-competent vectors: These are composed of attenuated viruses where genes that are not essential for replication in cultured cells *in vitro* are either mutated or deleted. Mutant viruses have been created that are non-neurovirulent e.g. 1716 (MacLean *et al.*, 1991). These can be produced to high titres and can hold multiple transgenes.

1.19 Cell culture models

Studies of motor neurons are quite difficult owing to the limitations in their isolation and culture. Also, no spontaneously arising motor neuron tumour cell line has ever been

described. In 1992 Cashman *et al.*, reported a proliferating, uniform cell line which constitutively expressed motor neuron attributes. These were developed by performing a series of fusions between motor neuron-enriched primary embryonic spinal cord cells and N18TG2 neuroblastoma cells. Unlike the parental N18TG2 cells, some of these neuroblastoma-spinal cord (NSC) cell lines constitutively express a variety of characteristics associated with primary neurons. Two of the cell lines isolated and used in this study are termed NSC-19 and NSC-34.

1.19.1 NSC-19 and NSC-34 cell lines

1.19.1.1 Motor neuron-like characteristics

NSC-19 and NSC-34 cells express many of the morphological and physiological properties of primary motor neurons i.e. they extend processes, establish contacts with cultured myotubes, synthesize and store acetylcholine, support action potentials, induce myotube twitching and express neurofilament proteins (Cashman *et al.*, 1992).

Hunter *et al.*, 1991, have shown that NSC-34 cells, similar to primary motor neurons and chick ciliary ganglion cells, adhere specifically to the leucine-arginine-glutamate (LRE) motif on S-laminin, a neuromuscular synapse-specific basal lamina glycoprotein (Hunter *et al.*, 1989 a, b; 1991). This adhesion provides the best evidence that this hybrid uniquely expresses motor neuron phenotypic characteristics, as this attribute was not observed in a large series of other neural cells lines (Hunter *et al.*, 1991). Thus NSC-34 is considered the most motor neuronal.

1.19.1.2 Ganglioside expression

Gangliosides are expressed in high concentrations in nervous tissues and are considered as cell surface antigens and receptors implicated in various biological functions including the control of cellular growth and differentiation (Yu and Saito, 1989). Due to the limitations and difficulties of using primary neurons, Matsumoto *et al.*, (1995), used various NSC cell lines *in lieu* of motor neurons, in an attempt to elucidate their ganglioside composition.

The major ganglioside of NSC-34 cells was GM2 (~75%). Also present was GD1a, with GM1 being present in small quantities. The high level of GM2 expression may be due to high expression of N-acetylgalactosaminyltransferase (EC 2.4.1.92) which converts GM3 to GM2. This pattern was entirely different from the other cell lines or the parental cell line i.e. NSC-19 cells expressed an unknown ganglioside, probably LM1, predominantly, with other gangliosides GD1a, GD1b, GM3, and a second unknown ganglioside, probably Hex-LM1 was also expressed.

The ganglioside expression of NSC-34 cells is thought to mirror most closely the characters of motor neuron cells i.e. the over representation of GM2 may correspond to a phenotypic property distinguishing motor neurons from other neurons. A paper published by Yoshino *et al.*, (1994) with GM2 in primary neurons is in agreement with this hypothesis.

1.20 Gangliosides

Gangliosides are complex glycolipids that are found in all cell membranes, especially in neuronal cells (Wiegandt, 1985). They consist of a ceramide tail with an oligosaccharide chain of up to four sugars that by definition must contain at least one sialic acid residue (Figure 1.3). Sialic acid is a generic name for N-acetylneuraminic acid, and it is almost always attached to the galactose group of the oligosaccharide chain. The ceramide is attached to glucose, which is followed by galactose-N-acetylgalactosamine-galactose linked to the oligosaccharide chain. This structure sits on the outer cell membrane with the ceramide anchored in the phospholipids, leaving the oligosaccharide chain exposed (Guido and Riboni, 1985).

The length and sialic acid composition of the extruding sugars from the plasma membrane, is thought to play an important role in the density, charge and binding characteristics of the cell (Ando, 1983). They possess high binding potential dependent on their ceramide and oligosaccharide portions (Tettamanti and Masserini, 1987). The gangliosides are believed to arrange themselves around membrane-bound proteins as

functional aggregates instead of a uniform distribution over the plasma membrane (Sharom and Grant, 1978; Yamakawa and Nagai, 1978). The interaction with these membrane-bound proteins is postulated to modulate the activity of the proteins and influence the membrane-mediated transfer of information (Fishman, 1988; Hakomori, 1990). Gangliosides have been postulated to have a multifunctional role, with influences on protein interactions (receptors, enzymes, ion channels and carriers) at the membrane level.

Studies have tried to identify differences in ganglioside composition between motor and sensory neurons, however no major differences in the localisation of the major gangliosides have been reported. However for gangliosides GM₁ and GD_{1a}, results have shown that the ceramide components in human sensory and motor nerves do significantly differ in their long chain fatty acids (Ogawa-Goto *et al.*, 1990). These differences can play a role in binding as Ogawa-Goto *et al.*, 1990 showed that anti-GM1 antibodies from sera of patients with Guillain-Barré Syndrome showed greater reactivity to sensory nerve GM₁ than motor nerve GM₁, and both of these showed greater reactivity with CNS GM₁ (Ohta, 1994). As the oligosaccharide chain and sialic acid are identical in these gangliosides the differences in reactivity are most likely due to the ceramide moiety, but could also be due to other factors in the local microenvironment.

Ganglioside

Structure

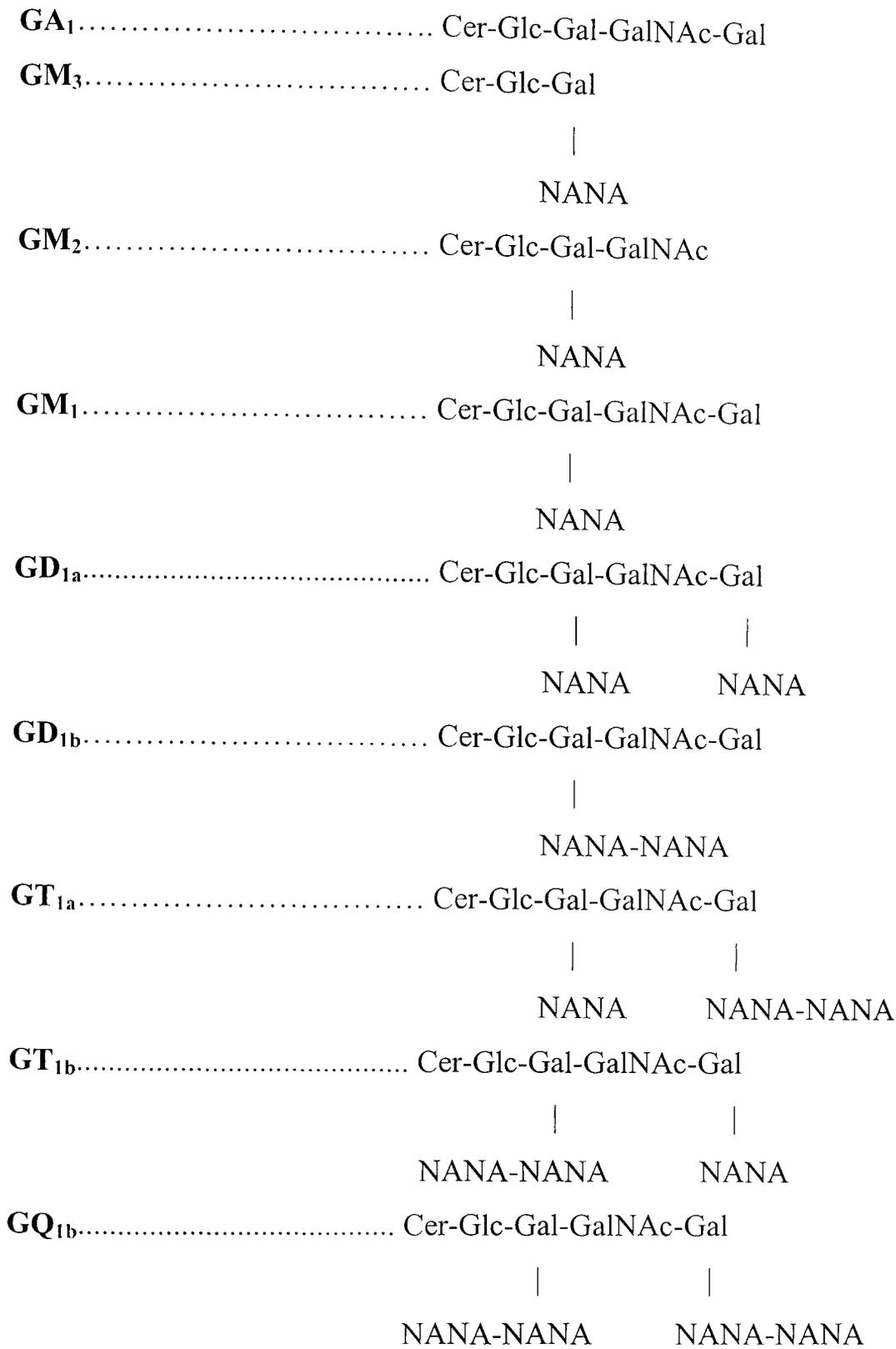


Figure 1.3 Structure of gangliosides and related compounds

Cer – Ceramide; Glc – glucose; Gal – galactose; GalNAc – *N*-acetylgalactosamine;
 NANA – *N*-acetylneuraminic acid (sialic acid)

1.21 *Escherichia coli* heat-labile enterotoxin (LT)

LT is an enterotoxin produced in the intestinal lumen by certain serotypes of *Escherichia coli*. It is responsible for a cholera-like illness in man. This is known by a variety of names including traveller's diarrhoea and "Montezuma's revenge" (Rowe *et al.*, 1970). It is an AB₅ hexamer consisting of 5 identical B subunits and a larger A subunit. The class of AB₅ toxins may be subdivided into families on the basis of sequence homology and catalytic activity. The cholera toxin (CT) family includes, in addition to CT itself, the *E. coli* heat-labile enterotoxins LT (Hol *et al.*, 1995) and LT-II (Pickett *et al.*, 1987; Pickett *et al.*, 1989), and a less-well characterised toxin from *Campylobacter jejuni* (Calva *et al.*, 1989) whose identity remains unproven.

1.21.1 Genetics

The gene encoding LT in *E. coli* is plasmid borne (So *et al.*, 1978; Dallas *et al.*, 1979). By analysis of deletion mutants (Dallas *et al.*, 1979) the genes for the LT subunits, LTA and LTB, termed *eltA* and *eltB*, were found to be transcribed into a single mRNA.

1.21.2 Structure

LTB is synthesized as a precursor with a N-terminal extension of 21 amino acids (Palva *et al.*, 1981). This leader peptide directs the protein through membranes and is cleaved from the polypeptide, releasing a mature protein.

CT and LT are heterohexameric proteins (*M_r*, 84,000) (Meckalanos *et al.*, 1983) produced by *Vibrio cholera* and *E. coli*, respectively. The toxins consist of an A subunit separated from the plane of a pentameric ring formed by 5 smaller, identical B subunits (Gill, 1976; Lai *et al.*, 1976; Ohtomo *et al.*, 1976; Gill *et al.*, 1981; Sixma *et al.*, 1991). Each B subunit interacts extensively with its adjacent subunits (Sixma *et al.*, 1993; Zhang *et al.*, 1995a; Zhang *et al.*, 1995b) via multiple hydrophobic interactions, Hydrogen-bonds and salt bridge interactions.

Consequently, B-subunit pentamers are highly stable, maintaining their quaternary structures in the presence of ionic detergents, in 8M urea, in 7M guanidinium chloride, and when heated to temperatures of $\leq 80^{\circ}\text{C}$. LTB pentamers are also stable over a pH range of 2.0-11.0, only undergoing disassembly at pH values less than 2.0 (Ruddock *et al.*, 1995).

The main features in the B-monomer are two three-stranded anti-parallel β -sheets, termed I ($\beta_2, \beta_3, \beta_4$) and II ($\beta_1, \beta_5, \beta_6$), a small N-terminal helix and a large “central” helix (Sixma *et al.*, 1991). A disulphide bridge (between amino acids 9 and 86) connects the N-terminal helix and strand β_5 (Sixma *et al.*, 1991). In the pentamer, the monomers are arranged such that sheet I of each subunit forms a six-stranded anti-parallel β -sheet with sheet II of the next subunit (Sixma, *et al.*, 1991). The pentamer has a diameter of $\sim 64\text{\AA}$ and a height of $\sim 40\text{\AA}$ (Sixma *et al.*, 1991).

Sixma *et al.*, (1991) have shown from crystallography that the A subunit can be described as a triangular or wedge shape in one view and a V-shape when rotated by 90° . Gill, D.M. (1976) by a series of cross-linking experiments showed that the one A subunit is actually composed of two peptides linked by a single disulphide bond. It is actually composed of two functionally different subunits, an enzymatic subunit (A1) and a short connector (A2) that mediates contact between A1 and the B pentamer. The A1 strand is a single-domain subunit with a complicated topology, consisting of many short stretches of secondary structure containing both α -helices and β -strands. The A2 subunit is an extended α -helix, which associates with A1 through extensive van der Waals interactions and continuing into the centre of the central pore formed by the B pentamer.

1.21.3 Mode of action

LT is produced in the intestinal lumen by *E. coli*. Both the A and the B subunits are synthesised intracellularly as precursor proteins. After translocation across the bacterial cytoplasmic membrane and removal of leader peptides, the AB_5 complex is assembled in the periplasm (Hirst *et al.*, 1984; Hofstra *et al.*, 1984). Enterotoxigenic strains of *E. coli*

may release the toxin as part of outer membrane fragments (Hirst *et al.*, 1984; Hofstra, *et al.*, 1984).

The B pentamer binds ganglioside GM1 in the membrane of intestinal epithelial cells, or any other cell that contains GM1 (van Heyningen, 1974) with its plane parallel to the membrane (Ludwig, *et al.*, 1986; Reed, *et al.*, 1987; Ribi *et al.*, 1988). Subunit A is inserted into the cytosol, activated by reduction of a disulphide bond (Gill and Rappaport, 1979; Mekalanos *et al.*, 1979; Tomasi and Montecucco, 1981) and associated with one or more cytosolic factors (Gill, and Meren, 1983; Kahn, and Gilman, 1984; Kahn and Gilman, 1986). The A1 fragment released by reduction is capable of binding NAD (Cassel and Selinger, 1977; Cassel and Pfeuffer, 1978) and catalysing the ADP-ribosylation of Gs α , a GTP-binding regulatory protein associated with adenylate cyclase. The result is a sharp increase in cyclic AMP (cAMP) production (Cassel and Pfeuffer, 1978; Gill, 1977; Gill and Richardson, 1980) which is responsible for the efflux of fluids and ions from the affected cell. The symptoms are manifested as a severe cramp and the copious “rice water” diarrhoea characteristic of the disease.

Neither the A subunit, nor the binding oligomer (B pentamer) is cytotoxic alone.

1.21.4 Binding of the B pentamer

The oligosaccharide moiety of GM1 is bound by the complete AB₅ hexamer and also by the B pentamer, but not by the monomeric B subunits (de Wolf *et al.*, 1981). There are five binding sites on the holotoxin, and binding of GM1 to the five sites is co-operative (Schön and Freire, 1989).

The binding mode of members of the CT family has been elucidated from X-ray structures of the LT AB₅ assembly (Sixma *et al.*, 1991; Sixma *et al.*, 1993) and a complex of the CT B pentamer with the branched pentasaccharide characteristic of GM1 (Merritt *et al.*, 1994).

The receptor-toxin binding interaction may be described as a “two-fingered grip” in which the Gal-GalNAc “forefinger” of the longer branch of the pentasaccharide is fairly deeply buried in the toxin, and the sialic acid “thumb” which constitutes the shorter branch, lies along the toxin surface. The terminal galactose residue of the GM1 is completely buried in the toxin-pentasaccharide complex; the binding site for this terminal sugar is notable for a complex net of H-bonding interactions tying all of the galactose hydroxyl oxygens to the protein, either directly or via tightly associated water molecules (Merritt *et al.*, 1994).

1.21.5 Binding specificities of LTB

Both CT and LT require the free carboxyl group of sialic acid for optimum binding (Schengrund and Ringler, 1989), since neither binds asialo-GM1 effectively. Nuclear magnetic resonance data (Sillerud *et al.*, 1981) suggest that a conformational change occurs in the oligosaccharide portion of the ganglioside when CT binds oligo-GM1. CT and LT bind the terminal sugar sequence Gal β 1-3GalNAc (NeuAc α 2-3) β 1-4Gal... (Holmgren *et al.*, 1973; Fukuta *et al.*, 1988), and LT can interact directly with lactose, binding the galactose portion of that sugar (Sixma *et al.*, 1992).

Both CT and LT bind ganglioside GM1. CT interacts very weakly, approximately 1,000-fold less effectively (Holmgren *et al.*, 1973), with GD_{1b}, but it does not bind any other related ganglioside. LT on the other hand interacts with a second class of receptors not recognised by CT (Holmgren *et al.*, 1982; Holmgren *et al.*, 1985; Griffiths *et al.*, 1986). These additional interactions include weak binding to GM2 and asialo-GM1 (Fukuta *et al.*, 1988).

1.21.6 LTB fusion proteins

Fusion proteins have been fused either genetically or chemically to the B-subunit (Czerkinsky *et al.*, 1989; Sanchez *et al.*, 1988; Dertzbaugh and Macrina, 1989; Nashar *et al.*, 1993). Fusions have been predominantly designed such that epitope or antigen extensions are present at the C-terminus of the B subunit (Nashar *et al.*, 1993) i.e. the 3

end of the gene. This is largely due to the fact that the C-terminus of LTB may be extended without interfering with B subunit assembly or GM1 binding. However, fusions with functional GM1 binding have also been constructed at the N-terminus (Dertzbaugh and Macrina, 1989).

Fusions that are too large or made at an inappropriate location may alter the conformation of LTB and hence its stability. Ruddock *et al.*, 1995, have shown that the acid stability of the pentamer is dependent upon the presence of an intersubunit bridge between the C-terminal carboxylate of the B subunit, Asn-103, and an adjacent subunit in the pentamer. Thus fusions with C-terminal extensions should disassemble at higher pH values than the wild type pentamer e.g. an (Asn-Ala-Asn-Pro)₃ extension at the C-terminus of hLTB (Fergusson *et al.*, 1990) results in a pentameric protein which disassembles below pH 3.52.

1.22 Translational differences between prokaryotes and eukaryotes

The mechanisms whereby ribosomes engage a messenger RNA and select the start site for translation differ between prokaryotes and eukaryotes. This initiation phase of protein synthesis does more than assemble the components that will assemble the components that will polymerise amino acids. Selection of the start codon sets the reading frame that is maintained normally throughout all subsequent steps in the translation process. Protein synthesis is often regulated at the level of initiation, which adds to the importance of that step.

1.22.1 Selection of start sites in prokaryotic mRNAs

At certain AUG or AUG-cognate codons, the small (30S) ribosomal subunit forms an initiation complex with a special form of tRNA (fMet-tRNA) and a GTP-binding protein called IF2. In addition to IF2, two other protein factors are required for initiation. The 8kDa IF1 is encoded by the *infA* gene in *Escherichia coli* and the 20kDa IF3 is encoded by *infC*. None of these initiation factors appears to interact directly with mRNA, although IF1 has an RNA binding domain (Sette *et al.*, 1997).

One function of IF1 and IF3 is to maintain a pool of free 30S subunits: IF1 promotes dissociation of 70S ribosomes, while IF3 binds to the 30S subunit (McCutcheon *et al.*, 1999) and prevents rejoining of the 50S subunit. Both of these factors also stabilize the binding of fMet-tRNA-IF2 to the 30S subunit. IF3 contributes to the fidelity of initiation in a special way by destabilizing initiation complexes that may transiently form at weak sites, such as non-AUG codons (Butler *et al.*, 1987, Sussman *et al.*, 1996). IF2 is the only tRNA-binding factor able to engage the 30S ribosomal subunit, and fMet-tRNA is the only tRNA recognised by IF2. Thus it follows that all polypeptide chains initiate with methionine. This conclusion also holds when the start codon is something other than AUG.

AUG is the most common initiator codon because it forms the most stable interaction with the CAU anticodon in fMet-tRNA, but GUG and UUG are used as start codons in >10% of bacterial genes (Blattner *et al.*, 1997; Cole *et al.*, 1998; Kunst *et al.*, 1997). A two base pair interaction is apparently sufficient for interaction with the anticodon with those non-AUG codons. The 30S subunit positions itself correctly on the mRNA, and the initiation phase is completed when the 50S ribosomal subunit joins, forming a 70S ribosome with fMet-tRNA occupying the P-site.

1.22.2 The Shine-Dalgarno (SD) interaction

Start codons in prokaryotic mRNAs are distinguished by an upstream, purine-rich sequence that pairs with a complementary sequence of the small ribosomal subunit. The earliest evidence for this SD interaction was the isolation of a complex between a 3' fragment of the 16S rRNA and a 30 nucleotide fragment of coliphage R17 mRNA that encompassed the start site for translation (Steitz and Jakes, 1975).

The SD sequence consists of three to nine contiguous bases in the mRNA that form standard base pairs (not including G-U) with some or all of bases 1534 to 1542 (ACCUCCUUA) at the 3' end of the 16S rRNA. Chen *et al.*, (1994) has shown that optimal translation occurs with a spacing of 5 nucleotides between the SD sequence and

the initiator codon. The SD sequence does function, albeit with reduced efficiency, when it resides as far as 13 nucleotides from the AUG codon (Chen *et al.*, 1994). Non-AUG codons that lack a SD sequence can support a very low level of initiation. This can be accomplished by coupled translation, i.e. an upstream cistron that terminates close to the UUG or GUG start codon of the next cistron delivers ribosomes to the internal start site which, lacking an SD element, is unable to recruit ribosomes independently (Groeneveld *et al.*, 1996). This device has been suggested to achieve limited expression of potent proteins that must not be overproduced.

The SD interaction augments initiation apparently by anchoring the 30S subunit in the vicinity of the start codon. By promoting ribosome entry while an mRNA is transiently unfolded, the SD interaction also counteracts the reformation of secondary structure that could hinder access to the AUG codon (De Smit and van Duin, 1994a). With many bacterial mRNAs, initiation depends simply on the SD sequence and nearby AUG codon being accessible to ribosomes (De Smit and van Duin, 1994b). There are however many mechanisms that control accessibility of the initiation codon, including repressor proteins, *trans*-acting RNAs, and switches in mRNA secondary structure (Brunel *et al.*, 1995; De Smit and van Duin, 1990; Lease *et al.*, 1998). In some cases, initiation is regulated by a repressor protein that traps the ribosome at the SD/AUG site, rather than blocking ribosome entry (Philippe *et al.*, 1993).

1.22.3 Selection of start sites in eukaryotic mRNAs

The eukaryotic mechanism of initiation is distinctive in that the small (40S) ribosomal subunit normally enters, not at the AUG codon, but at the 5' end of the mRNA. The 40S ribosomal subunit, carrying Met-tRNA_i-eIF2-GTP and other factors, then migrates through the 5' UTR until it encounters the first AUG codon, which is recognised by base pairing with the anticodon in Met-tRNA_i (Cigan *et al.*, 1988). When a 60S ribosomal subunit joins the paused 40S subunit, selection of the start codon is fixed.

In contrast to prokaryotic ribosomes, which often initiate at GUG or UUG, the eukaryotic initiation mechanism does not allow an alternative codon simply to substitute for AUG.

There is a limited ability to initiate at ACG or CUG in addition to the first AUG codon. As in prokaryotes, methionine is the first amino acid incorporated even when the initiator codon is something other than AUG (Yoon and Donahue, 1992). In the majority of natural eukaryotic mRNAs, the start site for translation is the first AUG codon, as the scanning model suggests (Kozak, 1987b). Flanking sequences modulate the efficiency with which the first AUG codon is recognised as a stop signal during the scanning phase of initiation. In vertebrate mRNAs, initiation sites usually conform to all or part of the sequence GCCRCCAUG, termed the Kozak sequence (Kozak, 1987a). This consensus has been derived from observing the sequences surrounding the first AUG in exons as well as mutagenesis studies. The most highly conserved position within this consensus sequence is the purine, usually A, in position -3 (3 nucleotides before the AUG codon, which is numbered $+1$ to $+3$). Mutations affecting A^{-3} strongly impair initiation *in vivo* and *in vitro* (Kozak, 1986; 1989). If an AUG codon is flanked by A^{-3} , or by G^{-3} and G^{+4} , the rest of the consensus sequence contributes only marginally. A recent study by Peri and Pandey (2001) have cast doubt over this consensus sequence. They analysed the context of initiator codons using a large dataset of curated human transcripts. When positions -3 and $+4$ are examined only 46% of transcripts contain a purine (A or G) at -3 and a G at $+4$. Thus, over half of the transcripts differ from what are believed to be the most conserved nucleotide positions (-3 and $+4$) surrounding the AUG. How this consensus sequence is recognised and how it functions are not yet known. One possibility is that an interaction with GCCACC might slow scanning and thus facilitate recognition of the AUG codon by Met-tRNA_i.

1.22.4 Alternative mechanisms

1.22.4.1 Leaky scanning

Leaky scanning means that some 40S ribosomal subunits bypass the first AUG codon and initiate instead at the second or, rarely, even the third AUG. The most predictable cause of leaky scanning is the absence of a good context around the first AUG codon. Leaky scanning also operates at a non-AUG codon, such as CUG, ACG or GUG. In higher

eukaryotes, ribosomes may initiate at an upstream non-AUG codon in addition to initiating at the first AUG, thus producing long and short forms of the encoded proteins; but a non-AUG codon normally cannot substitute for AUG as the sole start site (Kozak, 1991)

1.22.4.2 Reinitiation

After an 80S ribosome translates the first open reading frame (upORF) and reaches a terminator codon, the 40S subunit may hold on to the mRNA, resume scanning, and reinitiate at a downstream AUG codon. The ability of eukaryotic ribosomes to reinitiate is limited by the size of the upORF. In one study, the cut-off length was about 30 codons (Luukkonen *et al.*, 1995). This means one mRNA can produce a small peptide and a full-length protein but not two complete proteins. Reinitiation in eukaryotes is most efficient when the upORF terminates some distance before the start of the next cistron (Kozak, 1987b).

1.22.4.3 Internal initiation of translation

Internal ribosome entry sites (IRES) is the name given to a sequence that allows ribosomes to enter directly at an AUG codon rather than scanning from the capped 5' end of the mRNA (Jackson and Kaminski, 1995), allowing two transcripts to be translated from the same promoter.

1.23 Project rationale

The aim of this project is to develop several HSV-1 mutants with altered binding characteristics, such that the tropism of the virus can be changed so that it preferentially infects motor neurons. To achieve this, recombinant virus will be constructed from a gC-negative background to prevent HS binding. Gene fusions will be constructed between *E.coli* heat-labile enterotoxin B subunit (LTB) and varying lengths of gC. The size of the gC glycoprotein in both constructs will vary, however, none will contain HS-binding domains. LTB was chosen as it binds to several glycolipids including GM2. This is a

ganglioside that is expressed in several cell types in the body, especially in motor neurons. Thus, we hope that the mutant HSV following inoculation in the gastrocnemius muscle (a site which promotes motor neuron infection) will bind to GM2 ganglioside and infect motor neurons in greater numbers than wild type virus. Infection spread will be determined by β -gal expression. If successful this could act as a model for future vectors, with possible applications in gene therapy for treatment of several neurological diseases.

2. MATERIALS

2.1 Bacteria

The *E. coli* strain used for cloning was NM522. Bacteria were grown in L-broth (170mM NaCl, 10 g/l Difco bactotryptone, 5g/l yeast extract) and 2xYT (85mM NaCl, 16 g/l Difco bactotryptone, 5g/l yeast extract). Bacteria from glycerol stocks were plated out onto L-broth agar (L-broth containing 1.5% (w/v) agar).

2.2 Plasmids

pTRH101R was provided by Prof. T.R. Hirst. This is derived from plasmid pMMB138 (Sandkvist *et al.*, 1987) to which the *extB* gene had been cloned in the *EcoRI-HindIII* sites.

RL1.del was provided by Dr. E.A. McKie. This plasmid consists of the 5.9kb *BamHI k* fragment containing the RL1 gene cloned into the *BamHI* site of pGEM2fz (+). The 477bp *PflMI-BstEII* fragment (bases 125,292 to 125,769) was excised from the plasmid and replaced with a multi-cloning site, to form the plasmid RL1.del.

Plasmid *KpnI d*, provided by Dr. A.R. MacLean, contains the HSV-1 *KpnI d* fragment, cloned into the *PstI* site of pAT153 (Davison, 1981).

2.3 Cells and Tissue Culture Media

2.3.1 ETC10

Baby Hamster Kidney 21 clone 13 (BHK) cells (MacPherson and Stoker, 1961) were grown in Eagle's medium (Gibco) supplemented with 10% new-born calf serum (Gibco) and 10% (v/v) tryptose phosphate broth (Busby *et al.*, 1964).

2.3.2 DMEM10

Mouse embryo fibroblast 3T6 cells (European Tissue Culture Collection) and two Neuroblastoma-spinal cord (NSC) cell lines (NSC-19 and NSC-34) (Cashman *et al.*, 1992) were grown in Dulbecco's modified essential medium (DMEM) supplemented

with 10% foetal calf serum (DMEM10). Penicillin/streptomycin and/or gentycin were used at appropriate concentrations.

2.4 Viruses

2.4.1 Strain 17⁺

The wild-type parental virus used in this study was HSV-1 strain 17⁺ (Brown *et al.*, 1973).

2.4.2 Mutant gC-ve

A strain 17⁺ mutant variant with an in-frame stop codon disrupting UL44 (Cunningham and Davison, 1993) and with the gD promoter driving *lacZ* expression in the UL43 locus (A. MacLean, personal communication) was used for making recombinant viruses.

2.4.3 Mutant 1716

Strain 17⁺ deletion variant, 1716, was used as an ICP34.5 negative control in many experiments (MacLean, *et al.*, 1991).

2.5 Antisera

Mouse monoclonal antibodies against the monomeric form (LDS47 and LDS102) (Sandkvist *et al.*, 1987) and pentameric form (118-8) (Sandkvist *et al.*, 1987) of *E. coli* heat-labile enterotoxin were provided by Prof. T.R. Hirst. Dr. H. Marsden provided rabbit polyclonal antisera (4916 and 4901), which recognise gC. Antisera to the ORF of RL1, Rabbit 137, was provided by Dr. J. Harland (Brown *et al.*, 1997).

2.6 Radiochemicals

[³²P]dCTP radioisotope was supplied by DuPont NEN. It had an activity of 100μCi.

2.7 Enzymes

Restriction endonucleases were purchased from Promega and New England Biolaboratories. T4 DNA ligase was purchased from Promega. Lysozyme, Proteinase K and RNaseA were purchased from Sigma Chemical Company.

2.8 Sequencing

All sequencing in this thesis was carried out by DNAshef Ltd., Edinburgh.

2.9 Solutions

2.9.1 Bacterial Culture Reagents

Promega Wizard kit:

Resuspension Solution: 50mM Tris-HCl (pH7.5), 10mM EDTA, and 100µg/ml RNaseA

Lysis Solution: 0.2M NaOH, 1% (w/v) SDS

Neutralisation Solution: 1.32M KAc (pH4.8), 40% (v/v) propan-2-ol, and 4.2M guanidine hydrochloride

TE buffer: 10mM Tris-HCl (pH7.5), 1mM EDTA

Small scale preparation of plasmid DNA

Solution 1: 50mM Glucose, 10mM EDTA, 25mM Tris-HCl (pH8.0) and Lysozyme (4mg/ml)

Solution 2: 0.2M NaOH and 1% (w/v) SDS

Solution 3: 3M KAc (pH4.8)

2.9.2 Tissue Culture Reagents

PBS: 170mM NaCl, 3.4mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄ (pH7.2)

2.9.3 Agarose Gel Reagents

TAE: 0.04M Tris, 0.14% acetic acid (v/v), and 1mM EDTA (pH8.0)

TBE: 89mM Tris, 89mM boric acid, and 2mM EDTA

RE stop: 100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) Bromophenol blue in 5x TBE

2.9.4 Southern blot Reagents

Gel Soak 1: 200mM NaOH and 600mM NaCl

Gel Soak 2: 1M Tris-HCl (pH8.0) and 0.59M NaCl

20xSSC: 0.3M Na₃Citrate and 3M NaCl

Hybridization buffer: 0.54M NaHPO₄ (pH7.4) and 7.5% (w/v) SDS

Wash solution I: 0.5M NaHPO₄ (pH7.4), 7% (w/v) SDS

Wash solution II: 1% (v/v) 20xSSC, 0.1125% (w/v) SDS

2.9.5 SDS-PAGE Reagents

Boiling Mix: 151mM Tris-HCl (pH6.7), 6.28% (w/v) SDS, 0.15% (w/v) β -mercaptoethanol, 0.31% (v/v) glycerol, and 0.1% (w/v) bromophenol blue

Running Gel Buffer: 375mM Tris-HCl (pH8.9) and 0.1% (w/v) SDS

Stacking Gel Buffer: 0.1M Tris-HCl (pH6.7) and 0.1% (w/v) SDS

Tank Buffer: 6.32% (w/v) Tris, 4% (w/v) glycine, 1.33% (w/v) SDS

2.9.6 Non-denaturing PAGE Reagents

Boiling Mix: 151mM Tris-HCl (pH6.7), 0.31% (v/v) glycerol, and 0.1% (w/v) bromophenol blue

Running Gel Buffer: 375mM Tris-HCl (pH8.9)

Stacking Gel Buffer: 0.1M Tris-HCl (pH6.7)

Tank Buffer: 6.32% (w/v) Tris and 4% (w/v) glycine

2.9.7 Western Blot Reagents

Towbin buffer: 20% (v/v) MeOH, 25mM Tris-HCl (pH8.3), 192mM glycine

Fix and Stain: 46% (v/v) MeOH, 46% (v/v) dH₂O, 7.8% (v/v) acetic acid, 0.2% (w/v) coomassie brilliant blue R250

Destain: 5% (v/v) MeOH, 7% (v/v) acetic acid, 88% (v/v) dH₂O

2.10 Chemicals

Chemicals used were analytical grade and mostly obtained from Sigma Chemicals Company or BDH chemicals, UK.

APS and TEMED were supplied from Bio-Rad Laboratories

2.11 Other materials

2.11.1 Centrifuges

Volumes >50ml up to 10,000 rpm

Beckman Centrifuge

Volumes 1.5ml – 45ml up to 3,000 rpm

Benchtop Fison's Coolspin
Centrifuge

Volumes <1.5ml up to 13,000 rpm

Micro-centrifuge

2.11.2 Film

Autoradiograph XS-1 film (Southern and Western blots) was supplied by Kodak.

Autoradiographs were developed in an X-OGRAPH CompactX2.

Film used for immunofluorescence analysis was 400 ASA colour exposure from Fuji.

2.11.3 Plasticware

Plasticware was supplied mostly by eppendorf.

Tissue culture plates and dishes were supplied by Nunc.

2.11.4 Miscellaneous Equipment

DNA was crosslinked to nitrocellulose membrane using a XL-1000 UV crosslinker from the Spectronics Corp.

Optical densities were read using a Dynatech MR5000 plate reader.

3. METHODS

3.1 Tissue Culture

Herpes simplex virus growth was studied *in vitro* for protein expression and replication kinetics

3.2 Cells

Replication of HSV-1 strain 17⁺ is more efficient than RL1 deleted viruses in some (3T6) but not all cell lines (BHK) (Brown *et al.*, 1994a). Additional cell lines such as NSC-19 and NSC-34 were used to study viral adsorption and growth as these contain many phenotypes characteristic of motor neurons. These were used as a motor-neuron cell line *in vitro*.

3.3 BHK21/C13 Cells

Baby Hamster Kidney cells 21 (BHK) seeded at a 1 in 10 dilution were grown in 100ml ETC10 in 850cm² roller bottles in a humidified atmosphere of 5% CO₂ at 37⁰C for 3 to 4 days. Confluent cells (3x10⁸/roller bottle) were harvested as described in section 3.6.

3.4 3T6 Cells

3T6 cells were grown for 3 days in DMEM10 with 5% CO₂. Cells were passed in large flasks (T-175) by seeding at a 1 in 10 dilution in 50ml DMEM10. Confluent cells (3x10⁷ cells/T-175 flask) were harvested as described in section 3.6.

3.5 NSC-19 and NSC-34

NSC-19 and NSC-34 cells were grown for 3 days in DMEM10 with 5% CO₂. Cells were passed in large flasks (T-175) by seeding at a 1 in 10 dilution in 50ml DMEM10. Confluent cells (3x10⁷ cells/T-175 flask) were harvested as described in section 3.7.

3.6 Passaging BHK and 3T6 Cells

Trypsin solution was thawed from -20°C and mixed with HBSS to yield a Trypsin/HBSS solution.

T-175 flasks or roller bottles with confluent monolayers were opened in a category 2 hood, the supernatant decanted, and 10ml or 20ml, respectively, of HBSS poured over the monolayer and decanted. This was then repeated with HBSS/Trypsin and the solution left for 30 seconds before decanting. The cells were then shaken into 10ml or 20ml of media for further use.

3.7 Passaging NSC-19 and NSC-34 Cells

T-175 flasks with confluent monolayers were opened in a category 2 hood, the supernatant decanted and 20ml media added. The flask was then agitated to remove the cells. This cell suspension was then pipetted vigorously to break up clumps of cells before further use.

3.8 Cryopreservation of BHK and 3T6 Cells

Confluent cell monolayers of BHK or 3T6 cells were harvested from T175 flasks as described in section 3.6 by trypsinizing and resuspending in 15ml media and pipetted into Falcon tubes. Cells were centrifuged at 3,000g (Fison's Coolspin) at 4°C for 10 min and resuspended in 5ml of the appropriate growth medium, with 10% DMSO. Aliquots of 1ml were pipetted into 1.5ml cryo-vials. These were frozen overnight at -70°C and moved to a liquid nitrogen freezer for long term storage.

3.9 Cryopreservation of NSC-19 and NSC-34 Cells

For cryopreservation of NSC cells, cell media of 80% heat-inactivated FCS and 20% DMEM with 1% penicillin/streptomycin added was prepared by filtering through a $0.22\mu\text{m}$ filter. The filtered cell media was then stored on ice. Freezing media was prepared by filtering 70% heat-inactivated FCS, 10% DMEM with 1% penicillin/streptomycin through a $0.22\mu\text{m}$ filter, with 20% DMSO added afterwards. The freezing media was then stored on ice.

Confluent cell monolayers were harvested from T175 flasks as described in section 3.7 and pipetted into falcon tubes. Cells were centrifuged at 3,000g (Fison's Coolspin) at 4⁰C for 10 min. The supernatant was discarded and the pellet was resuspended in 3ml of filtered cell media. 0.5ml cell suspension was aliquoted into a chilled 1.5ml cryo-vial. 0.5ml freezing media was then slowly dripped into each vial using a chilled pipette, with care being taken not to agitate the tube. These were frozen overnight at -70⁰C and moved to a liquid nitrogen freezer for long-term storage

3.10 Growth and harvest of HSV

Confluent roller bottles were infected with 0.02 pfu/cell of HSV in 20ml of ETC10, assuming that there were 2x10⁸ cells per roller bottle. These were incubated at 37⁰C for 3-4 days until cpe was complete. Cells were then shaken into the medium. The cells were pelleted in 250ml plastic falcon tubes by spinning at 2000g for 10 min at 4⁰C. The supernatant and cell pellet were separated and two individual virus stocks were prepared:

Supernatant stock: The supernatant was poured into 250ml centrifuge bottles and spun at 12000g for 2 hours (4⁰C) in a Sorvall GSA rotor. The supernatant was discarded, and the virus pellet resuspended in 1ml ETC10 or PBS/10% NCS per roller bottle. The pellet was sonicated until homogenous, before aliquoting into 1.5ml amounts and storing at -70⁰C.

Cell-associated stock: The cell pellet was resuspended in 0.5ml medium/roller bottle and thoroughly sonicated before spinning at 2000g for 10 min at 4⁰C. The supernatant was kept on ice while the process was repeated. The two supernatants were combined to give the cell-associated virus stock. This was aliquoted and stored as above.

3.11 Titration of virus stocks

Virus stocks were serially diluted 10-fold in PBS/10% NCS. 100µl aliquots were added to 70% confluent monolayers of BHK21/C13 cells on 60mm petri dishes from which the medium had been removed. The plates were incubated at 37⁰C for 45 min-1 hour, to allow adsorption of the virus to the cells before overlaying with 4ml EMC10%. Plates were incubated at 37⁰C for 2 days. Monolayers were fixed and stained with Geimsa at

RT for 1h. After washing, plaques were counted on a dissection microscope and virus titres were calculated as pfu/ml.

3.12 Preparation of HSV DNA

For preparation of a large scale HSV DNA stock, 10 roller bottles of almost confluent monolayers of BHK21/C13 cells were infected with virus at a moi of 0.02 pfu/cell. The infection was continued at 37⁰C until cpe was extensive (3-4 days). The cells were shaken into the medium and spun at 2000g for 10 min. The supernatant was kept and stored on ice. The cell pellet was resuspended in 10ml 0.5% (w/v) NP40 in RSB (10mM KCl, 1.5mM MgCl₂, 10mM Tris-HCl, pH 7.5) and left on ice for 10 min. The nuclei were pelleted by centrifuging at 1000g for 3 min (4⁰C), with the supernatant kept. This was repeated, and the supernatants were pooled and spun at 12K for 2 hours in a Sorvall centrifuge to pellet the virus.

The virus pellet, containing cell released and cytoplasmic virus was resuspended in NTE buffer (10mM Tris-HCl, pH7.5, 10mM NaCl, 1mM EDTA) and sonicated until no lumps remained. EDTA and SDS were added to a final concentration of 10mM and 2% (w/v) respectively to cause lysis of the virus. Viral DNA was extracted 3-4 times with saturated phenol and once with chloroform:isoamylalcohol, prior to precipitating with 2 volumes of ethanol at RT for 5 min. DNA was pelleted at 2000g for 10 min, washed with 70% ethanol, dried at 37⁰C and resuspended in a minimal volume of H₂O (containing RNaseA 50µg/ml).

3.13 One-cycle Replication Kinetics

30mm plates were seeded with 2x10⁶ cells/plate in 2ml ETC10 growth medium and cells incubated overnight at 37⁰C and 5%CO₂. Growth media was poured off and 100µl of the virus (0.01-0.001pfu/cell) added. The plates were returned to 37⁰C and 5% CO₂ for 1h. After 1 hour the plates were overlaid with 2ml ETC10. This was time point 0h. Plates were incubated at the appropriate temperature and harvested at the designated time points (usually 0, 4, 8, 24, 48 and 72h) by scraping the cell monolayer into the medium and transferring the suspension to a sterile bijou bottle. The samples were sonicated and

stored at -70°C until the experiment was complete. The samples were quickly thawed and titrated as described in section 3.11.

3.14 Large scale isolation of plasmid DNA: Promega © Wizard™ Maxiprep

A 500ml *E. coli* plasmid-containing culture was grown overnight in L-broth containing 100 $\mu\text{g/ml}$ ampicillin. Cells were pelleted by centrifugation at 5,000rpm (Sorvall GSA rotor) for 10 min. at RT. The supernatant was discarded and the pellet resuspended in 15ml resuspension solution. 15ml Lysis Solution was added to the pellet by gently mixing throughout and incubating at RT for 20 min. 15ml Neutralization Solution was added and mixed by inverting several times. The samples were centrifuged at 2,000g (Fison's Coolspin) for 20 min. The clarified supernatant was poured into a fresh 50ml Falcon tube and 0.5x propan-2-ol was added and mixed by inversion. Samples were centrifuged at 2,000rpm (Fison's Coolspin) for 15 min at RT. The supernatant was discarded and the pellet left to dry. Once dry, the pellet was resuspended in 2ml TE buffer. 10ml Wizard™ resin was added to the pellet. This solution was added to a Wizard™ maxi-column. A vacuum was applied to draw the solution through the Wizard™ maxi-column. The resin was washed by adding 80% EtOH to the Wizard™ maxi-column. The DNA was eluted by adding 1.5ml of preheated H₂O ($65-70^{\circ}\text{C}$) and incubating for 1 min at RT before centrifuging the Wizard™ maxi-column at 2,500g (Fison's Coolspin) for 5 min. A final step of purification of DNA was done by using a syringe with a Luer-Lok® extension and 0.22 μm filter. Once the solution went down through the filter the DNA was precipitated by adding 2 volumes EtOH and 0.1 volume 3M NaAc, vortexing and placing on dry ice for 20 min before centrifuging at 2,500g (Fison's Coolspin) for 10 min. The supernatant was discarded and the pellet was rinsed with 1ml 70% EtOH and centrifuged as above. The supernatant was discarded and the pellet left to dry at RT. The DNA pellet was resuspended in 1.5ml dH₂O (containing 50 $\mu\text{g/ml}$ RNaseA).

3.15 Restriction endonuclease digestion

Plasmid and viral DNA were routinely evaluated by different restriction endonuclease digestion profiles. The duration and temperature conditions were carried out according to the manufacturers' instructions. Enzyme activity was stopped by placing RE stop in the reaction mixture. Digestion was visualized by electrophoresis through an agarose gel and staining with ethidium bromide.

3.16 Phenol chloroform extraction and precipitation of plasmid DNA

An equal volume of phenol:chloroform was added to the DNA, mixed by vortexing and subjected to centrifugation for 2 min at 13,000g at RT (microfuge). The aqueous layer containing the DNA was pipetted into a fresh tube. Two volumes of EtOH and 0.1 volume 3M NaAc was added to the aqueous layer, vortexed, and placed on dry ice for 20 min. The solution was centrifuged for 20 min at 13,000g (microfuge). The supernatant was discarded and 1ml 70% EtOH added and centrifugation carried out as stated above. The supernatant was discarded and the DNA pellet left to dry. The pellet was resuspended in dH₂O (containing 50µg/ml RNaseA).

3.17 Ligation of plasmid DNA

Different molar ratios of plasmid DNA to vector were added to a total volume of 14µl. 4µl 5x ligation buffer was added, the solution mixed and centrifuged to collect at the bottom of the tube. Lastly, 2µl T4 DNA ligase (3U/µl) was added to the solution, mixed well and placed in a water bath at 16°C overnight. The following day this was used in a transformation reaction.

3.18 Transformation of *E. coli*

10-15µl of a glycerol *E. coli* stock was used to inoculate 10ml 2YT medium. This was grown overnight at 37°C with shaking to produce a saturated culture. 1ml of this culture was then used to inoculate 100ml of 2YT, and this was incubated at 37°C for 2 ½ hours with shaking. The bacterial cells were pelleted by centrifugation at 2000g for 10 min at

4°C. The cells were resuspended in 1/10th volume of TSB. These cells were incubated on ice for at least 10 minutes, after which time they were considered competent for transformation.

1-10µl of DNA was mixed with 100 µl of competent bacteria and incubated on ice for 30 min. The volume was made up to 1ml by the addition of TSB and the bacteria further incubated for 1 hour at 37⁰C. If the bacteria had been transformed with a plasmid which conferred a particular trait e.g. antibiotic resistance, the 1 hour incubation at 37⁰C allowed plasmid gene expression to occur.

100µl of the transformed bacteria were plated out onto L-broth agar plates containing, if appropriate, 100µg/ml ampicillin. Plates were allowed to dry at room temperature, before incubating in an inverted position at 37⁰C overnight.

3.19 Small scale preparation of plasmid DNA

Single, transformed bacterial colonies were inoculated into 5ml 2YT containing 100µg/ml ampicillin. These were incubated at 37⁰C for 8-16 hours with vigorous shaking. 1.5ml of this culture was removed and the bacteria pelleted by low -speed centrifugation (6000g, 2 min). The pellet was resuspended by vortexing in 100µl of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8, 4mg/ml lysozyme added just prior to use). After five minutes at RT 200µl of solution II was added (0.2M NaOH, 1%SDS) and the cultures mixed by rapid inversion. Following 5 min incubation at RT 150µl of solution III was added (5M KAc, pH4.8). Following 5 min incubation at RT, a white precipitate consisting of the cell debris was pelleted at high speed (13K) in a microcentrifuge.

Plasmid DNA was extracted from the supernatant using an equal volume of phenol:chloroform, followed by ethanol precipitation at RT for 1 min. The DNA was pelleted by spinning at 13,000g for 5 min in a microfuge, washed in 70% ethanol, dried in a speedivac and resuspended in 100 µl H₂O (containing RNaseA 50µg/ml).

3.20 Agarose gel electrophoresis

Agarose gels (0.6-1.5% (w/v) agarose) were prepared by boiling the appropriate quantity of agarose in 1xTBE or 1xTAE. Ethidium bromide was added at a concentration of 0.5µg/ml, immediately prior to pouring the gel. The solution was poured into a tray (BioRad) which held a comb. Once cool, the tray was placed in an electrophoresis kit, the comb removed and samples pipetted into individual wells. Electrophoresis of the samples was carried out until the dye front was approximately 1cm from the end of the gel and visualised using a short wave UV lamp. A 100bp or 1kb marker of known concentration was run alongside the fragments to enable confirmation of the vector/fragment size and approximate quantitation of the concentration of each sample.

3.21 Minigel electrophoresis

This method was generally used to quantitate small volumes of fragment and vector DNA prior to ligation. Gels were prepared by boiling the appropriate concentration of agarose in 1xTBE. When cool, 0.5µg/ml ethidium bromide was added and the agarose was poured directly into the minigel kit (Bio-Rad). Once set, the comb was removed and 50ml 1xTBE was added to the kit. Samples (generally (10µl) were run at 50V for 1hour and visualised using a short wave UV lamp. A 100bp or 1kb marker of known concentration was run alongside the fragments to enable confirmation of the vector/fragment size and approximate quantitation of the concentration of each sample.

3.22 Elution of Restriction Enzyme Fragments from Agarose Gel

DNA was digested with the appropriate restriction enzyme and electrophoresed through an agarose gel containing 0.5mg/ml EtBr until the fragment to be isolated was well resolved. The gel was visualized under a short wave UV light and the desired fragment excised using a sterile scalpel. The DNA was eluted using the Nucleon DNA purification kit. A 1.5ml microtube was weighed and the gel slice added to the tube. This was re-weighed with the DNA volume calculated as 1µl equivalent to 1mg. 4.5 volumes of standard salt was added to the DNA sample before incubating at 55⁰C to melt the gel slice. Once melted, 10µl of resin was added to the tube. This was incubated at RT for 1

min with frequent inversions before centrifuging at 13,000g for 30 seconds. The supernatant was discarded and 1ml of 1x wash added to resuspend the pellet. This was centrifuged at 13,000g for 30 seconds. The wash solution was discarded, and the tube was left with its lid open at RT for 5 min to remove any remaining ethanol droplets. The DNA was eluted by adding 20µl of sterile water to the tube, incubating at RT for 1 min before centrifuging at 13,000g for 30 seconds. The eluted DNA sample was removed into a new labeled tube.

3.23 Transfection of virus DNA by CaPO₄ and DMSO boost

Viral HSV DNA and plasmid DNA were diluted to the desired molar ratios and placed in an eppendorf tube. The following were added to the DNA, 400µl HEBS (pH7.5) and calf thymus DNA (10µg/ml). These were mixed and spun down in a microfuge and CaCl₂ added to a final concentration of 130mM. The samples were left at RT for 30 min and added to a fresh confluent monolayer of BHK cells in 60mm plates, from which the medium had been removed. Following incubation at 37°C for 1h, cells were overlaid with 4ml ETC10 and incubated at 37°C. Four hours later, the media was removed from the plates and the plates washed twice with ETC10. For exactly 4 min the cells were overlaid with 1ml 25% (w/v) DMSO in HEBS at RT. 4ml ETC10 was added immediately and subsequently the plates were washed twice with 4ml ETC10, and overlaid with 4ml ETC10. Plates were incubated at 37°C until cpe was complete. Cells were scraped into the medium and pelleted by centrifugation at 2,000g (Fison's Coolspin) for 10 min, resuspended in 1ml medium, sonicated and the virus was stored at -70°C until further use.

3.24 Transfection of mammalian cells

Promega Transfection Kit:

In a sterile eppendorf tube, 6-12µg DNA was added to 37µl 2M CaCl₂, with dH₂O added to a final volume of 300µl. This was vortexed before slowly adding to 300µl 2X HBS. This was incubated at RT for 30 min to allow the formation of a calcium phosphate-DNA co-precipitate, before being added dropwise to a fresh confluent monolayer of BHK cells

in 60mm plates, from which the medium had been removed. Following 4h at 37°C, the DNA complex was removed and 1ml 10% DMSO/PBS (v/v) added. After 2.5 min the DMSO solution was removed and 4ml ETC10 added. Plates were then incubated at 37°C for 48h.

3.25 Plaque purification

Once cpe was complete in transfection plates (section 3.23), cells were scraped into growth media, transferred to a bijou bottle and sonicated in a soni-bath until homogenous. Serial 10-fold dilutions were made in PBS/10% NCS. 100µl from the 10⁻² to 10⁻⁶ dilutions from each were plated on fresh BHK monolayers in 60mm plates from which the medium had been removed. After 1h adsorption at 37°C and 5% CO₂, the plates were rinsed once with PBS/10% NCS and overlaid with 4ml EMC10 and incubation continued at 37°C for a further 48-72h. Using a 200µl Gilson pipette individual plaques from the plate with the most well-separated plaques were picked, resuspended in 500µl PBS/10% NCS and sonicated in a soni-bath until homogenous. This procedure was either repeated or a Southern blot performed to check the purity of the recombinant virus.

3.26 DNA Extraction of HSV Plaque Isolates

During viral purification, viral plaque isolates were routinely checked for purity by DNA digestion with subsequent Southern blotting. Plaque isolates were harvested as described above. 200µl of the viral suspension was used to infect a linbro well of BHK cells. The virus was incubated at 37°C until cpe was evident. The medium was removed and 200µl of cell lysis buffer was added (20mM Tris (pH7.5), 2mM EDTA, 0.5mg Proteinase K and 0.5% (w/v) SDS) to the infected monolayer. This was incubated for at least 6h at 37°C. The lysate was transferred to an eppendorf tube containing 15µl 5M NaCl and 200µl phenol:chloroform (1:1), vortexed and centrifuged at 13,000g for 2 min. The top layer was removed and added to 200µl of chloroform, vortexed and centrifuged at 13,000g for 2 min. The top layer was again removed and the DNA pelleted by adding to 1ml of EtOH, vortexing and centrifuging at 13,000g for 5 min. The pellet was washed with 70% EtOH, before air-drying and dissolving in 200µl dH₂O containing RNase A (50µg/ml).

This was digested with a suitable restriction enzyme before checking for viral purity by Southern blotting (section 3.28).

3.27 Radiolabelling DNA

25ng template DNA in dH₂O in a final volume of 13.5µl was placed in a tube and denatured by heating in a boiling water bath for 10 min followed by chilling quickly in an ice bath. The DNA was pulsed in a microcentrifuge and placed on ice while 5µl 5x random primer buffer, BSA, dNTPs (minus dCTP) and 2U Klenow was added. 2.5µl (50µCi[α³²P]dCTP) was added and the tube incubated for at least 1h at 37°C. The reaction was stopped by placing the tube in a boiling water bath for 10 min. After boiling the radiolabelled probe was used with the blot as described in section 3.28.

3.28 Southern Blot and Hybridisation

Purified DNA was digested with the appropriate restriction enzyme(s) before electrophoresing on a 1% (w/v) agarose gel. The gel was visualised under a short wave UV light to confirm DNA digestion, then placed in a bath containing gel soak I for 30 min at RT. After rinsing with dH₂O the gel was incubated in gel soak II for 30 min at RT and rinsed as above. Finally, the gel was incubated in 20xSSC for 30 min at RT. A pack of "Hi-Dry" towels were stacked on the bench followed by three sheets of dry Whatmann 3MM paper. On top were 3 sheets of pre-soaked 3MM paper and 1 sheet of nitrocellulose membrane (Hybond-N) in 20xSSC. The gel was placed on top. A glass plate and heavy weight were placed atop and this was left overnight to transfer DNA. To transfer DNA to two membranes, this procedure was repeated on top of the gel. The following day, the DNA was cross-linked to the membrane by a UV crosslinker. The membrane was allowed to dry and placed in a glass bottle with hybridisation buffer and radiolabelled-DNA probe added. The membrane was incubated in a Hybaid oven at 65°C overnight. The membrane was washed three times in wash solution at 65°C for 30min. Finally, it was dried. The membrane was exposed to XSI film making sure it was 100% dry each time it was exposed.

3.29 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed in 12.5% gels using 2.5% (w/w) crosslinker. Slab gels were cast vertically in a sandwich consisting of 2 glass plates separated by a 1.5mm thick perspex spacer. Typically, 60ml gel mix was prepared using a running gel buffer (RGB) which contained 375mM Tris-HCl, pH 8.9 and 0.1% (w/v) SDS. Polymerisation was achieved by the addition of 0.06% (w/v) ammonium persulphate (APS), and 0.04% (v/v) N, N, N', N', tetramethylethylenediamine (TEMED), just prior to pouring. A thin layer of butan-2-ol (3-5ml) was poured on top to exclude air and enable polymerization of the gel. Prior to adding the stacking gel, the butan-2-ol was removed and the surface of the running gel was rinsed several times with distilled water. It was then thoroughly dried using filter papers to allow good adhesion between it and the stacking gel.

The stacking gel was composed of 5% acrylamide crosslinked with the same ratio of N,N'-methylbisacrylamide used in the resolving gel, in a buffer (SGB), composed of 0.11M Tris-HCl, pH 6.7 and 0.1% (w/v) SDS. As previously, APS and TEMED were added to the gel just prior to pouring and a teflon comb was used to form the wells. Samples were boiled for 10 minutes in a sample buffer (151mM Tris-HCl, pH 7.6, 6.28% (w/v) SDS, 0.15% (v/v) 2-mercaptoethanol, 0.31% (v/v) glycerol) before loading on the gel, and were run for 3-4 hr at 55mA.

Gels were fixed or stained in a solution of methanol: acetic acid: water, 50:7:50, with or without 0.2% (w/v) Coomassie Brilliant Blue R250, for 1 hr at RT, then destained for a minimum of 3x 30 minutes in a 5:7:88 solution of methanol: acetic acid: water.

3.30 Non-denaturing Polyacrylamide Gel Electrophoresis

Protein samples whereby interactions between proteins were to be examined, for example those of the pentameric form of LTB, were analysed using non-denaturing PAGE. These gels were prepared in a similar manner to those of SDS-PAGE (section 3.29), however none of the RGB, SGB, sample buffer, and tank buffer contained SDS (section 2.9.6). Protein samples were loaded onto the gel without boiling and separated at 55mA for 3-4h.

3.31 Western Blotting

SDS-PAGE was carried out as previously described and Western blotting carried out essentially as described by Towbin (1979). In general two types of protein sample were used for Western blotting. The first was infected cell proteins which had been harvested into boiling mix at a density of 10^7 cells/ml, and the second was small aliquots of fractions which had been obtained from protein purification columns. After separation of the proteins, they were transferred onto nitrocellulose using a Bio-Rad blotting apparatus. The foam pads, sheets of Whatmann 3MM paper and the nitrocellulose to be used for the transfer were pre-soaked in transfer buffer (192mM glycine, 25mM Tris-HCl pH 8.3, 20% methanol). The gel was placed on top of the nitrocellulose, 3 sheets of 3MM paper and one foam pad. Three further sheets of 3MM paper and foam pad were then placed on top. The plastic folder was then closed, placed in the transfer kit and blotted at 250mA for a minimum of 3h. After this time the nitrocellulose was removed, placed in a plastic "tuperware" tub and then blocked for 2x 30 minutes using 2% dried milk in PBS, 0.05% Tween 20. The nitrocellulose was washed for 3x 10 minutes in PBS, 0.05% Tween 20, 1% BSA, before addition of the first antibody diluted appropriately in PBS, 0.05% Tween 20, 1% BSA. Following incubation at 37⁰C for 2h or overnight at 4⁰C the nitrocellulose was washed as before. The second antibody, normally goat anti-mouse IgG coupled to HRP, was added at 1/1000 dilution in PBS/complete, 0.05% Tween 20, 1% BSA at 37⁰C for 1h, after which time the nitrocellulose was washed twice with PBS/0.05%Tween 20 and once with PBS alone.

The protein-antibody complexes were then visualised using Amersham ECL reagents, according to manufacturer's recommended instructions.

3.32 Protein Expression in Infected Cell Extracts

Virus stocks were used to infect 60mm plates containing 4×10^6 cells with 5-10pfu/cell. Plates were incubated for the desired time at 37⁰C. Samples were harvested by removing media and washing infected cell monolayers twice with cold PBS. The monolayers were taken off in 300 μ l 1xBM.

3.33 Harvesting Protein Samples for Denaturing Gels

Protein samples from infected cell monolayers or transfected cells were often concentrated using a MICROSEP™ microconcentrator. The proteins were harvested by removing the medium from a 60mm plate and washing the cells with warmed PBS. The cells were then scraped off into 10ml of PBS and centrifuged at 1,500g for 10 min. The pellet was resuspended using 600µl of buffer (50mM Hepes pH7.5, 50mM NaCl, 10% glycerol, 0.5% NP40) before sonicating briefly, and centrifuging at 13,000g for 2 min to remove cellular debris. The supernatant was stored at -20°C.

3.34 Concentration of Protein Samples

Several protein samples which were thought to contain low concentrations of a protein were concentrated using a MICROSEP™ microconcentrator (Pall Gelman Laboratories). Protein samples were harvested as per section 3.33 or 3.34, depending on whether the concentrated sample was to be run on a denaturing or non-denaturing PAGE gel. The samples were then loaded onto a MICROSEP™ microconcentrator, with a molecular weight cutoff 3 to 6 times less than the molecular weight of the protein to be retained, typically 3kDa or 10 kDa, and centrifuged at 7,500g (Sorvall centrifuge) for 90 min. The concentrated sample (30µl) was then stored at -20°C until loading on a protein gel.

3.35 Harvesting Protein Samples for Non-denaturing Gels

Protein samples from virally infected monolayers or from transfected cells were often separated by non-denaturing PAGE. To avoid disassociation of the proteins the media was removed from a 60mm plate and the cells washed with cold PBS. The cells were then scraped into 10ml of PBS and centrifuged at 1,500g for 10 min. 300µl of buffer (20mM Hepes pH7.6, 75mM NaCl, 10% glycerol, 0.4% Triton X100) was used to resuspend the pellet, which was then incubated on ice for 30 min. Cellular debris was removed by centrifuging at 13,000g for 2 min. Immediately 300µl of non-denaturing sample buffer was added and the protein sample was loaded, without boiling, onto a non-denaturing gel (section 3.30).

3.36 Indirect Immunofluorescence

Linbro wells containing coverslips were seeded at $0.5-5 \times 10^5$ cells/well and incubated overnight at 37°C with $5\% \text{CO}_2$. The cells were infected at a low moi (0.1 pfu/cell) for 16h, or transfected with plasmid DNA. The cells were fixed by removing the medium and washing three times with PBS and adding fixing solution, usually paraformaldehyde or methanol, for 10 min RT or 10 min at -20°C , respectively. Coverslips were washed three times in PBS and blocked using PBS/1%BSA for 1h at 4°C . Again coverslips were washed three times with PBS. The primary antibody was diluted to the appropriate dilution in PBS/1%BSA and $100\mu\text{l}$ added to each coverslip. Coverslips were incubated in the primary antibody for 2h at 4°C . Following this, excess antibody was removed and the coverslips washed three times with PBS. The secondary antibody was diluted to the appropriate dilution in PBS/1%BSA and $100\mu\text{l}$ added to each coverslip. These were incubated for 1h at RT and washed three times with PBS.

Coverslips were mounted by dipping in H_2O and drying on a paper towel at RT until dry. A small drop of mounting fluid was placed on a glass slide and the dry coverslip, cells facing down, placed on the drop. A longer period of storage required painting clear nail varnish around the edges of the coverslips to prevent drying.

To detect cell surface antigens the cells were unfixed prior to antibody staining. Antibody staining was carried out as above. Following the removal of unbound secondary antibody the coverslips were treated with ice-cold MeOH for 10 min at -20°C , then washed three times with PBS and once with H_2O before mounting.

3.37 X-gal Staining

Viruses and plasmids containing the β -galactosidase gene *lacZ* were identified on BHK cells by staining with X-gal. Cells that were infected with a *lacZ*-containing virus or transformed with a *lacZ*-containing plasmid were rinsed with PBS. Cells were fixed using 2% formaldehyde, 0.2% paraformaldehyde in PBS for 5 min at RT. The fixing solution was removed and cells rinsed with PBS, before the addition of staining solution (5mM Potassium Ferrocyanate, 5mM Potassium Ferricyanate, 2mM MgCl_2 , 2.5% 20mg/ml X-gal). Plates were incubated at RT for 16 hours to allow development of the blue colour.

3.38 Viral Adsorption to Cells

30mm plates were seeded with 2×10^6 cells/plate in 2ml growth medium and incubated overnight at 37°C and $5\% \text{CO}_2$. Growth media was poured off and 500pfu virus, containing *LacZ*, (in 100 μl) was added to each plate. This was time point 0 min. Plates were incubated at 37°C , and at designated time points, usually 0, 2.5, 5, 15, 30 and 60 min, any unbound virus was removed, the plates washed several times with warmed PBS and 2ml of growth medium added. The plates were incubated at 37°C for 24h to allow expression of the β -galactosidase gene. The plates were then stained for X-gal and the number of blue cells were counted on a dissection microscope, whereby every blue cell represented one bound virus

3.39 HSV Glycolipid-Binding ELISA

Currently all the ligands to which HSV binds are unknown. Nervous tissue is enriched with gangliosides, and these may be HSV binding ligands. Thus to determine how wild-type HSV and the LTB-containing mutant viruses bind to these sugars, if at all, a glycolipid-binding ELISA was developed. An Immulon 2 96-well plate (Dynex) was coated with various gangliolipids, at the following dilutions in MeOH.

	Rows	Columns	
Blank Wells	A,B,C,D	1, 2	100 μl MeOH
GM1	A,B,C,D	3, 4	100 μl GM1 2 $\mu\text{g}/\text{ml}$
GM2	A,B,C,D	5, 6	100 μl GM2 2 $\mu\text{g}/\text{ml}$
GM3	A,B,C,D	7, 8	100 μl GM3 2 $\mu\text{g}/\text{ml}$
GA1	A,B,C,D	9, 10	100 μl GA1 2 $\mu\text{g}/\text{ml}$
GD1a	A,B,C,D	11, 12	100 μl GD1a 2 $\mu\text{g}/\text{ml}$
GD1b	E,F,G,H	1, 2	100 μl GD1b 2 $\mu\text{g}/\text{ml}$
GT1b	E,F,G,H	3, 4	100 μl GT1b 2 $\mu\text{g}/\text{ml}$
GQ1b	E,F,G,H	5, 6	100 μl GQ1b 1 $\mu\text{g}/\text{ml}$
GD3	E,F,G,H	7, 8	100 μl GD3 2 $\mu\text{g}/\text{ml}$

The MeOH was allowed to evaporate in a fume hood until the plate dried, before storing the plate at 4⁰C. The plate was then blocked by the addition of 150µl PBS-2%BSA to each well for 1h at 4⁰C. The PBS-BSA was discarded by flicking and the plate dried by clapping against a paper towel. Virus was diluted to a desired concentration in PBS-1%BSA and 100µl added to each well for 1h at 4⁰C. The virus was discarded and the plate washed in PBS/0.05% Tween. 100µl of primary antibody, Rabbit α-HSV (1:200) (Dako) was added at 4⁰C for 2h before washing with PBS/0.05% Tween. 100µl of secondary antibody, Donkey α-Rabbit (1:100) (Dako) was added for 1h at 4⁰C before washing with PBS/0.05% Tween. 100µl of tertiary antibody, PAP (1:100) (Dako) was added for 1h at 4⁰C, before washing with PBS/0.05% Tween. The plate was then developed by the addition of 100µl of substrate solution (15mg O-phenylenediamine tablet dissolved in 60ml of a citrate buffer (0.02M citric acid, 0.05M Na₂HPO₄), activated by the addition of 20µl of 30% H₂O₂) to each well. After 10 min in the dark, the reaction was stopped by the addition of 50µl 2M H₂SO₄ to each well. The optical densities of the wells were then read at 490nm.

3.40 Heparin-binding ELISA

To determine how wild type and various HSV mutants bind to heparin an ELISA was developed. 100µl of a 2µg/ml heparin solution, in PBS, was added to each well and stored at 4⁰C overnight. The plate was then blocked by the addition of 150µl PBS-2%BSA to each well for 1h at 4⁰C. The PBS-BSA was discarded by flicking and the plate dried by clapping against a paper towel. Virus was diluted to a desired concentration in PBS-1%BSA and 100µl added to each well for a selected time, normally 1, 3, 5, 15, 30, 60 min., at 37⁰C before washing in PBS/0.05% Tween. Bound virus was detected as in section 3.39.

3.41 *In vitro* Transcription

Using the Ambion (Europe) Ltd. MEGAscript T7 Transcription Kit (Cat No: 1334), RNA was synthesised from the DNA template: pGEM.LTB.gC(b) as described:

Preparation of Template DNA

Ten micrograms of plasmid pGEM.LTB.gC(b) was linearised by *Bam*HI digestion to prepare a template that would generate transcripts from the gene fusion LTB.gC(b). The restriction digest was terminated by adding 1/20 volume of 0.5M EDTA and 1/10 volume of 5M sodium acetate and then treated with Proteinase K (200µg/ml) and SDS (0.5%) for 45 minutes at 50⁰C to remove residual RNase. DNA was extracted from the supernatant using an equal volume of phenol:chloroform pH 8.0 followed by precipitation with two volumes of 100% 'AnalaR' grade ethanol at -20⁰C for 1 hour. DNA was pelleted by centrifugation at 13,000g for 30 minutes at 4⁰C, washed once with 70% ethanol, dried and resuspended in nuclease-free H₂O at a concentration of 0.5µg/µl.

Transcription Reaction

One microgram of the linearised plasmid template was used in a 20µl transcription reaction, assembled in a microcentrifuge tube, as described in the manufacturers protocol. The reaction was incubated at 37⁰C for 4 hours prior to degrading the template DNA by the addition of 1µl of RNase-free DNase I and a further incubation at 37⁰C for 15 minutes. The reaction was stopped and the RNA precipitated by the addition of 30µl of nuclease free H₂O and 25µl of Lithium Chloride precipitation solution: 7.5M Lithium Chloride, 50mM EDTA, mixing thoroughly and chilling the reaction at -20⁰C for 1 hour. RNA was pelleted by centrifugation at 13,000g for 30 minutes at 4⁰C, washed once with 70% ethanol, dried and resuspended in 20µl of nuclease-free H₂O.

Analysis of Transcription Products by Gel Electrophoresis

The RNA transcript (4.5µl) and 2µg RNA Century-Plus size markers [Ambion (Europe) Ltd., Cat No: 7145] (2µl marker + 2.5µl nuclease free H₂O) were electrophoresed on a 6% Acrylamide/7M Urea gel in 1x TBE buffer. An equal volume of gel loading buffer (50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol

FF) was added to the RNA, the samples heated to 80⁰C for 10 minutes to denature any secondary structure, chilled on ice, centrifuged briefly and loaded directly onto the denaturing polyacrylamide gel. RNA was visualised by ethidium bromide staining.

RNA Century-Plus Size Markers Information

The RNA size standards are a set of 7 RNA transcripts of 100, 200, 300, 400, 500, 750 and 1000bp.

4. CONSTRUCTION OF HSV-1 RECOMBINANT VIRUSES CONTAINING LTB-gC GENE FUSIONS

4.1 Introduction

HSV-1 predominantly infects sensory neurons, however it is capable of infecting motor neurons (Dobson *et al.*, 1990). Infection by HSV-1 involves an initial adsorption of the virion at the cell surface, followed by virion penetration of the cell. From studies on epithelial cells, the principal virion protein involved in adsorption is glycoprotein C (gC) which binds to cellular glycosaminoglycan, heparan sulphate (HS) (Fuller and Spear, 1985; Herold *et al.*, 1991; Svennerholm *et al.*, 1991). gC has been characterised, and two domains of the protein have been shown to play a role in adsorption to HS - one including Arg-143, -145, -147 and Thr-150, and the other containing Gly-247 (Trybala *et al.*, 1994). Though separated by almost 100 amino acids, in the tertiary structure of the protein they are in close proximity. These regions are Arg-rich and of a polycationic nature. Other polycationic substances e.g. neomycin, poly-L-lysine are known to compete with HSV attachment to cells (Langeland *et al.*, 1987, 1988, 1990; WuDunn and Spear, 1989; Campadelli-Fiume *et al.*, 1990).

Gangliosides are complex glycolipids that are found in all cell membranes. These are found especially in neuronal cells (Wiegandt, 1985). Due to the limitations and difficulties of using primary neurons, Matsumoto *et al.*, (1995), used various NSC cell lines *in lieu* of motor neurons, in an attempt to elucidate their ganglioside composition. Due to various phenotypic characteristics e.g. adhesion to the leucine-arginine-glutamate (LRE) motif of S-laminin, a neuromuscular synapse-specific basal lamina glycoprotein (Hunter *et al.*, 1989 a, b; 1991), NSC-34 was seen to be the most motor neuron-like cell line available. The major ganglioside of NSC-34 cells was GM2 (~75%). Also present was GD1a, with GM1 present in small quantities.

Escherichia coli heat-labile enterotoxin (LT) is an enterotoxin produced by *E. coli*. It is an AB₅ hexamer consisting of 5 identical B subunits and a larger A subunit. The toxins consist of an A subunit separated from the plane of a pentameric ring formed by 5

smaller, identical B subunits (Gill, 1976; Lai *et al.*, 1976; Ohtomo *et al.*, 1976; Gill *et al.*, 1981; Sixma *et al.*, 1991). The pentameric B subunit binds to GM1 in the membrane of intestinal epithelial cells, or any other cell that contains GM1 (van Heyningen, 1974).

Thus, to promote binding towards gangliosides present in motor neurons and shift the balance of tropism of HSV towards motor neurons, two recombinant viruses were constructed, each containing separate truncated portions of gC fused to LTB.

4.2 Construction of Mutant Viruses: RFa and RFb

Two new recombinant viruses were constructed in a gC-ve (LacZ) background (Cunningham and Davison, 1993) and named RFa and RFb. These viruses each contain fusions of a different length of the gC gene (both with the HS-binding domains deleted) to LTB. To generate these viruses, several plasmids were sub-cloned and are described in detail below (Figure 4.1).

4.3 Subcloning of etxB

The *E. coli* LTB gene was contained in the plasmid pTRH101R (Prof. T. Hirst.). This was subcloned into pGEM2fz(+) (Promega). The 400bp *EcoRI* - *SpeI* fragment containing the LTB gene was cloned into the *EcoRI* / *XbaI* sites of pGEM-3Zf(+) to create the plasmid pGEM.LTB (Figure 4.2).

4.4 Creation of gC-LTB fusions

Based on the findings of Holland *et al.*, 1988, the gC gene was altered such that the encoded protein would retain its cytoplasmic domain. This ensured that the truncated glycoprotein (and any fusions to it) will be anchored in the plasma membrane, and will also be inserted into the virion envelope. Two varying lengths of gC gene were constructed: one encoding the cytoplasmic and transmembrane domains (a.a.s:478-511), termed gC(b), the other encoding the cytoplasmic and transmembrane domains and also a region of the extracellular domain that contains Cys-5 to Cys-8 (a.a.s:377-511), termed gC(a). The smaller of the two gene sizes was chosen as it encodes the smallest portion of glycoprotein C that will be inserted into the membrane (Holland *et al.*, 1988), and also

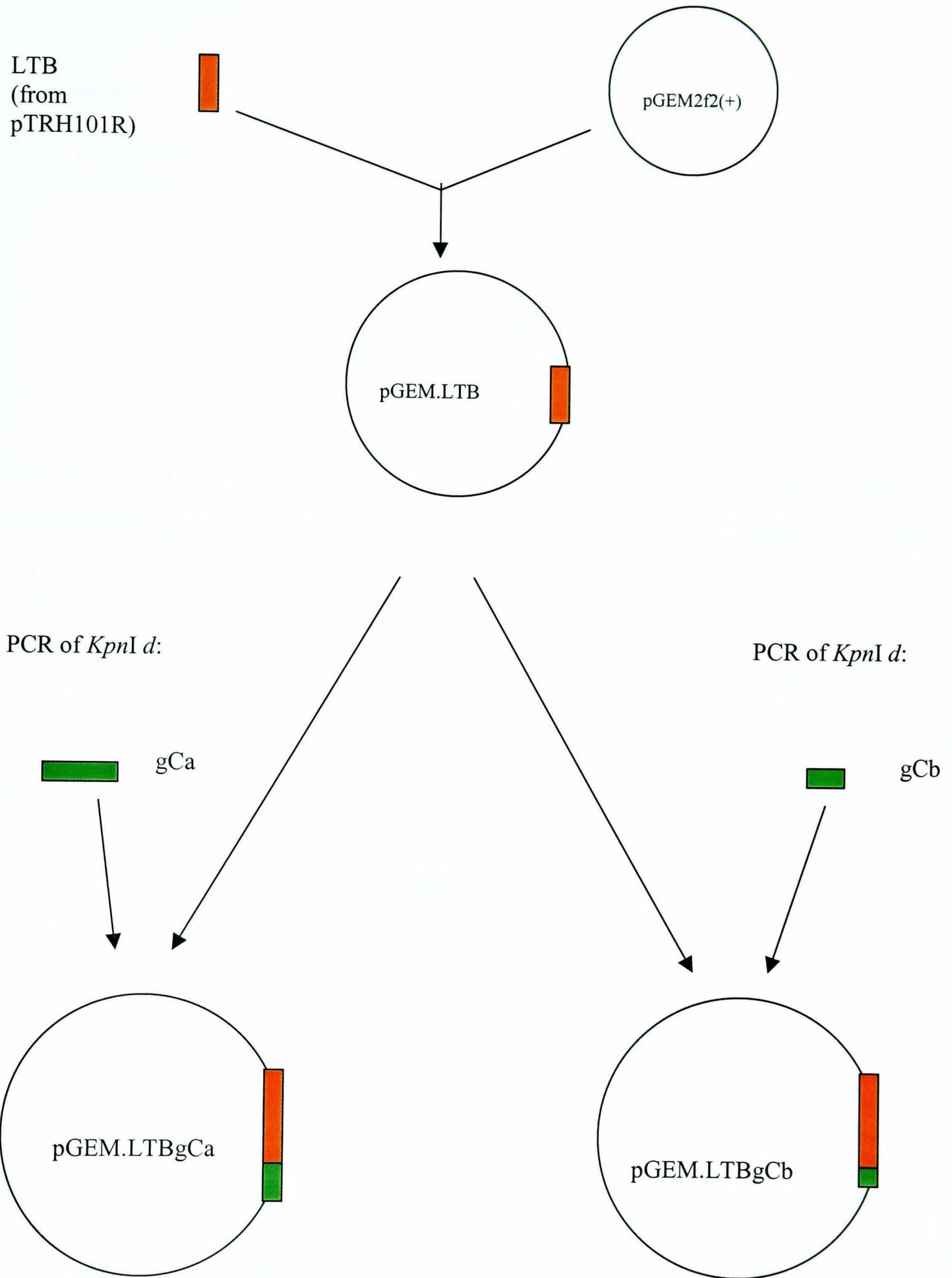


Figure 4.1 Schematic overview of cloning steps (part 1)

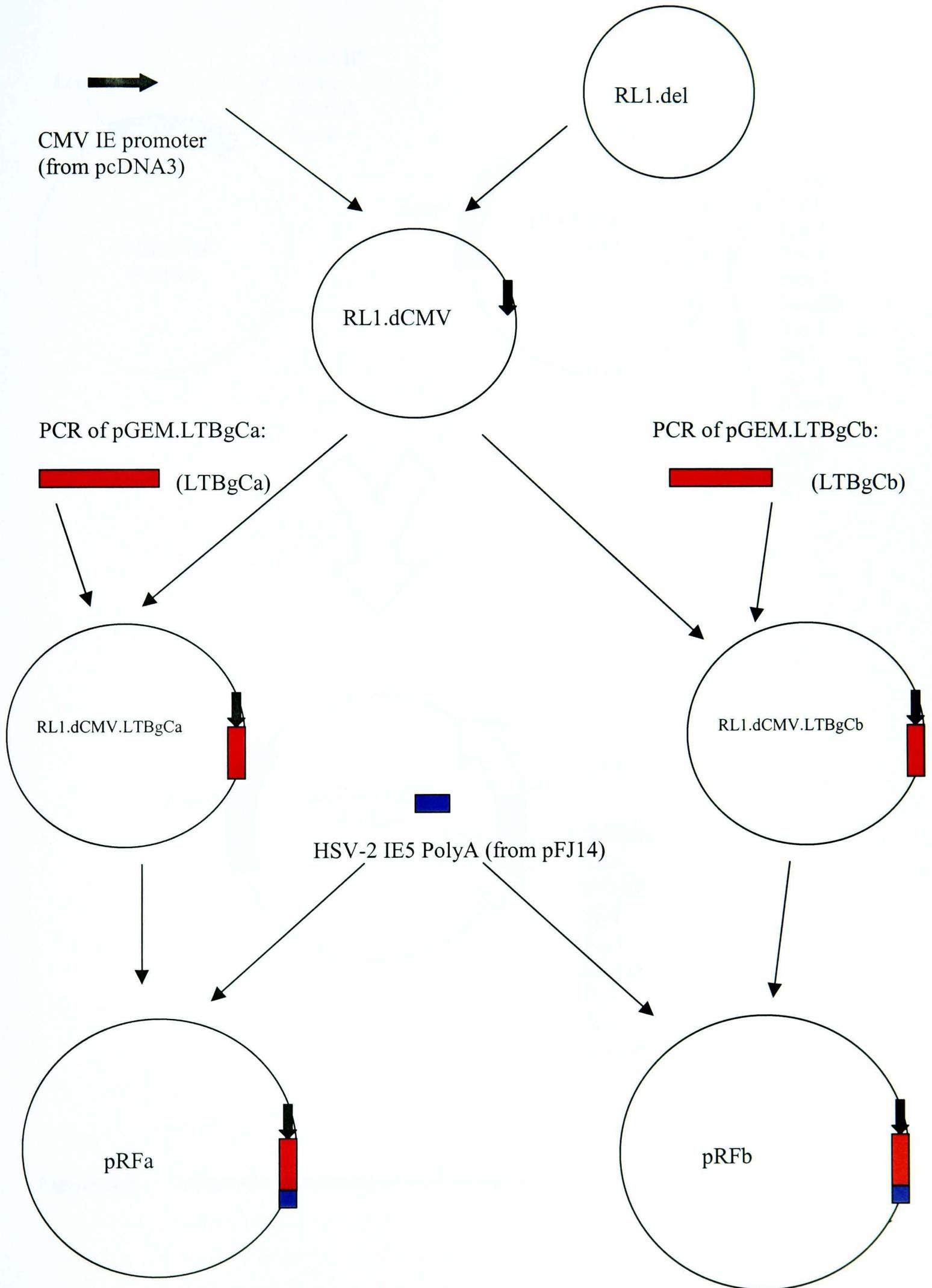


Figure 4.1 Schematic overview of cloning steps (part 2)

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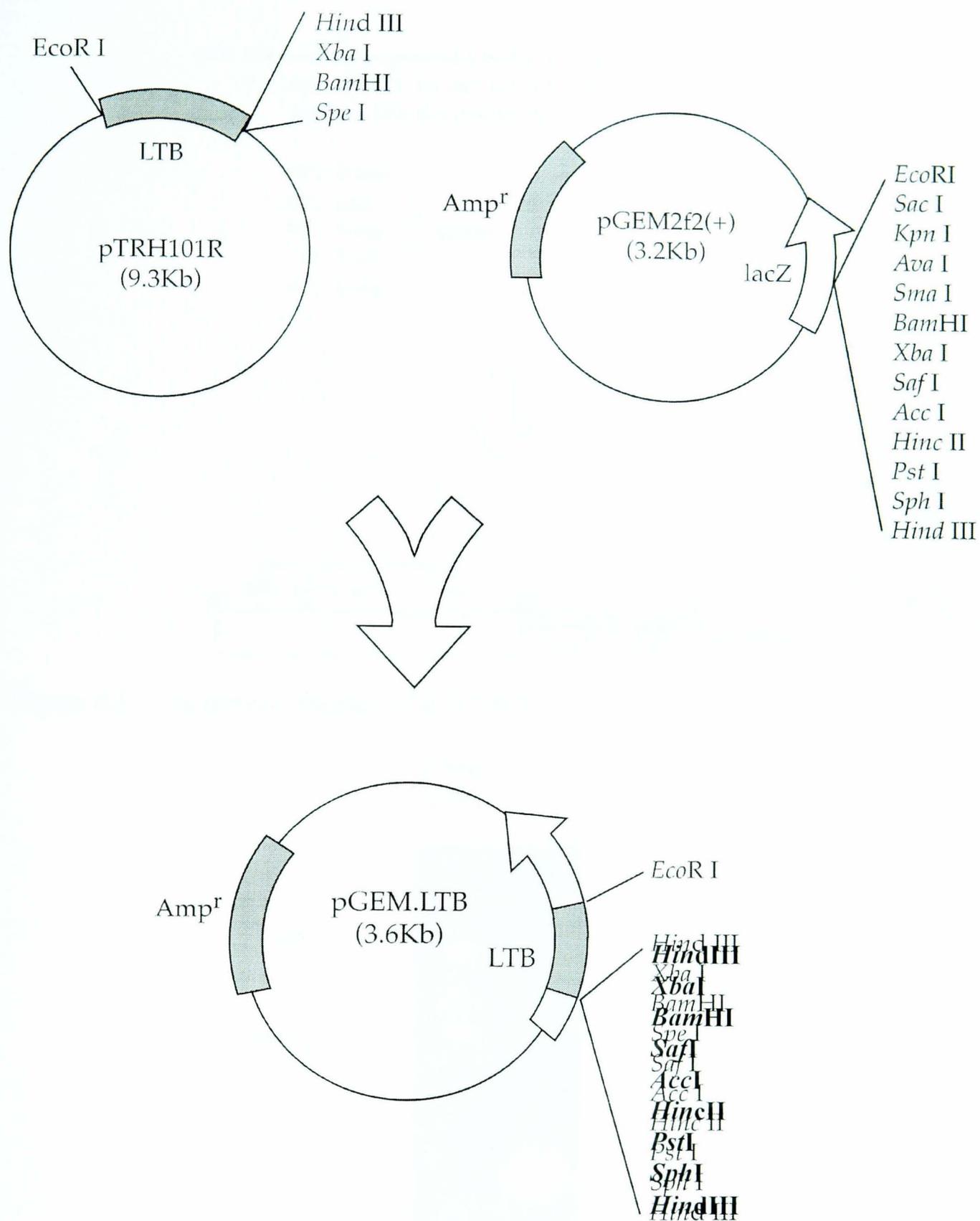


Figure 4.2 Schematic subcloning of *extB* into pGEM2fz(+)

gC(a) PCR - amplified using *Kpn I* d as a template and primers:

```

5' GGG TAA GCT TTT ACC GCC GAT GAC G 3'
5' AAC CAA GCT TAT GGA ATT TGG GGT C 3'

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98°C - 10 mins
98°C - 1 min
57°C - 30 secs
72°C - 30 secs
72°C - 10 mins

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} 30 cycles

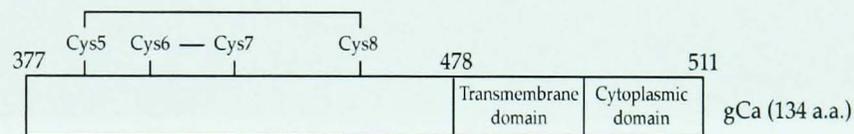
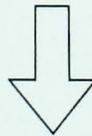


Figure 4.3 Schematic diagram of gC(a) PCR

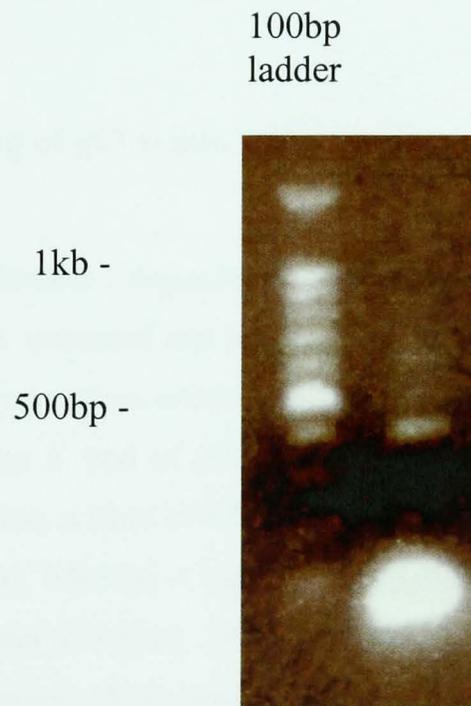


Figure 4.4 PCR-amplification of gC(a)

Using *Kpn I* d as a template, the 425bp fragment was amplified using primers GC1 and GC3. Following the PCR reaction, the reaction mix was fractionated on a 1% agarose gel. The 425bp gC fragment (as indicated by arrow) was then cloned into pGEM.LTB.

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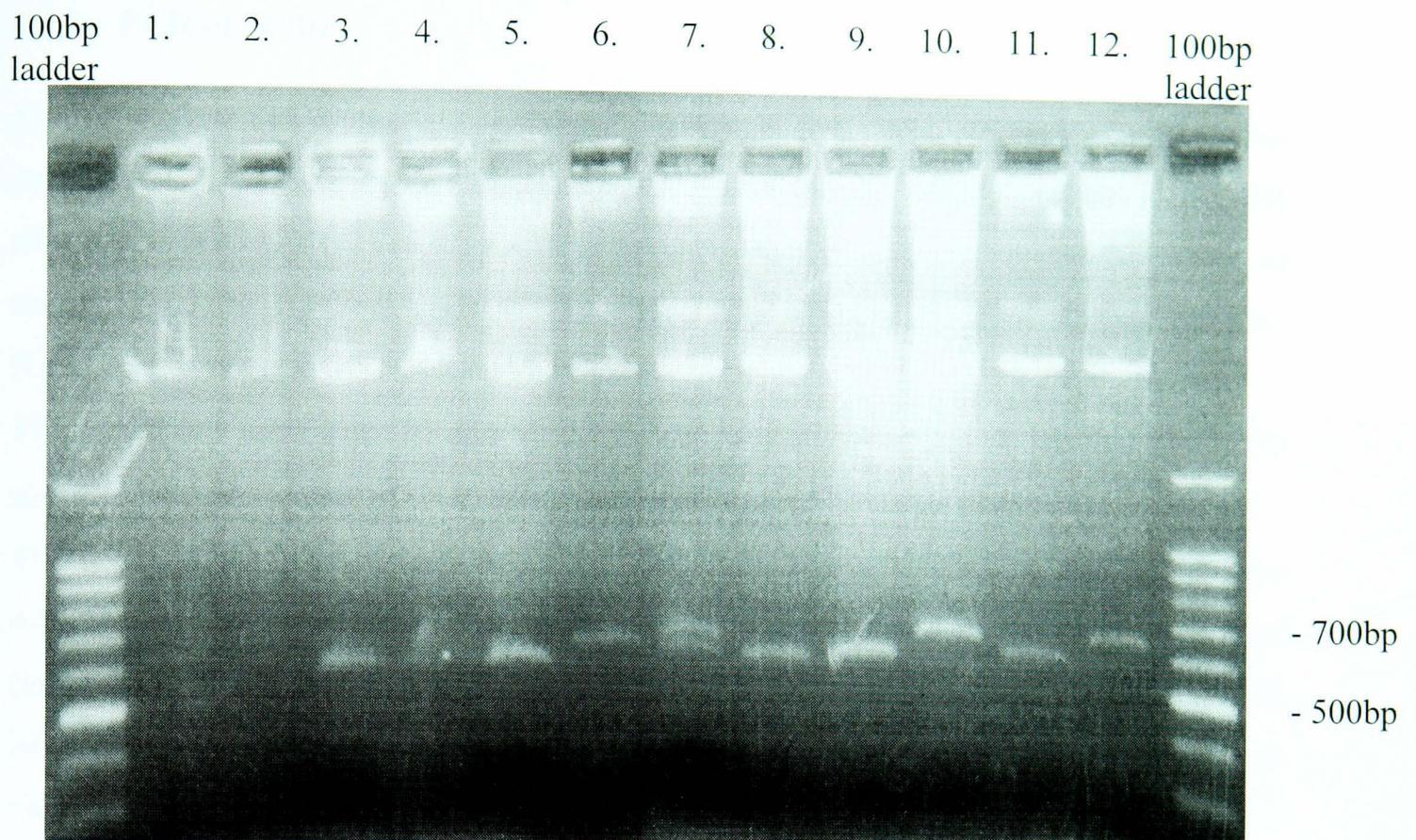


Figure 4.6 Cloning of gC(a) into pGEM.LTB

Following ligation of *Hind*III - digested gC(a) into the *Hind*III site of pGEM.LTB, 12 colonies had their plasmid DNA extracted and analysed by *Eco*RI/*Xba*I digestion (lanes 1-12) on a 1% agarose gel. An *Xba*I restriction enzyme site exists at base-pair position 97,669 of the HSV-1 genome, 229bp from the 5' end of gC(a). Thus, as the gC(a) insert could recombine into the plasmid in either direction, a band of 629bp indicates the insert is in the correct orientation (with a further band of 3.4kb), whereas a band of 564bp signifies the incorrect orientation of the insert (with a further band of 3.5kb). From the diagram, several clones formed with insertions in either directions. However, clone number 10, that showed a band of 629bp, was chosen and grown up to provide plasmid pGEM.LTB.gC(a).

4.4.2 PCR of gC(b)

KpnI d was used as a template, and the gC fragment was amplified using the following primers: GC4, a 25mer which anneals to the 3' end of the gC gene, and contains a *Bam*HI site: (GGGTGGATCCTTACCGCCGATGACC), and GC2, a 25mer, which anneals to the gC gene from amino acids 478 to 481. This contains an *Xba*I recognition site: (GGAGTCTAGATGGGTGGGGATTGGA)

The conditions used were 10 min at 98° C, followed by 30 cycles of 1min at 98° C, 30 sec at 65° C, and 30 sec at 72° C, with final extension at 72° C for 10 min (Figure 4.7).

The 125bp gC(b) fragment was amplified, fractionated on a 1% TAE agarose gel (Figure 4.8), excised using a Nucleon gel extraction kit, then digested with *Bam*HI and *Xba*I (these sites contained within the PCR primers). The fragment was cloned into the *Bam*HI, *Xba*I sites of pGEM.LTB to generate plasmid pGEM.LTBgC(b) (Figure 4.9). Upon cloning, the gene fusion remained in-frame:

+100				+478
ATC AGT ATG GAA AAG CTT TCT AGA TGG GTG GGG				
LTB gene product	<i>Hind</i> III	<i>Xba</i> I		gC(b)

4.5 Creation of an expression plasmid

In order for the mutant viruses to be avirulent, the glycoprotein-toxin fusions were inserted into the RL1 locus. It's gene product, ICP34.5, has been shown to be one of the major determinants of virus pathogenicity. Following intracranial inoculation, mutants in RL1 are 100,000 less virulent than wild type viruses (Taha *et al.*, 1989; Chou *et al.*, 1990; MacLean *et al.*, 1991). In order to achieve homologous recombination of the gene fusions into the RL1 locus, the fusions were cloned into the plasmid RL1.del (E.A. McKie). This plasmid consists of the 5.9kb *Bam*HI *k* fragment containing the RL1 gene cloned into the *Bam*HI site of pGEM2fz(+). The 477bp *Pf*IMI-*Bst*EII fragment (bases 125,292 to 125,769) was excised from the plasmid and replaced with a multi-cloning site, to form the plasmid RL1.del (Figure 4.11). This plasmid was then manipulated further as described below.

gC(b) PCR - amplified using *Kpn Id* as a template and primers:

5' GGA GTC TAG ATG GGT GGG GAT TGG A 3'
5' GGG TGG ATC CTT ACC GCC GAT GAC C 3'

98°C - 10 mins

98°C - 1 min

65°C - 30 secs

72°C - 30 secs

30 cycles

72°C - 10 mins

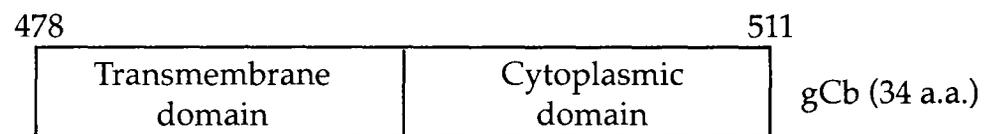
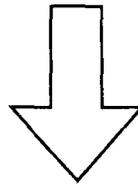


Figure 4.7 Schematic diagram of gC(b) PCR

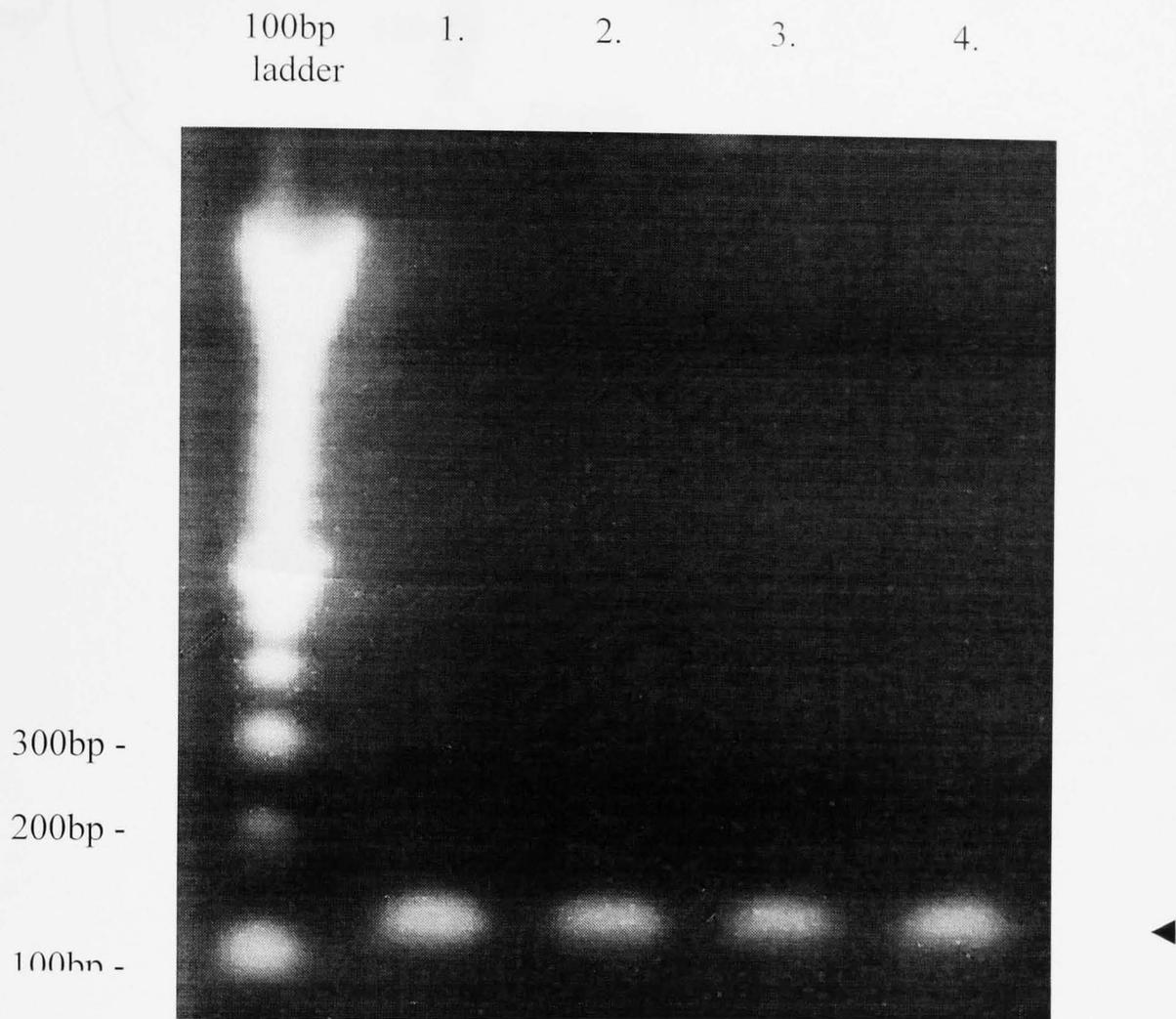


Figure 4.8 PCR-amplification of gC(b)

Using *KpnI d* as a template, the 125bp fragment was amplified using primers GC2 and GC4. Four various concentrations of primer and template were used in the PCR mix, and the reactions were fractionated on a 1% agarose gel. As can be seen from the diagram, all four lanes show amplification of the 125bp fragment (indicated by arrow)

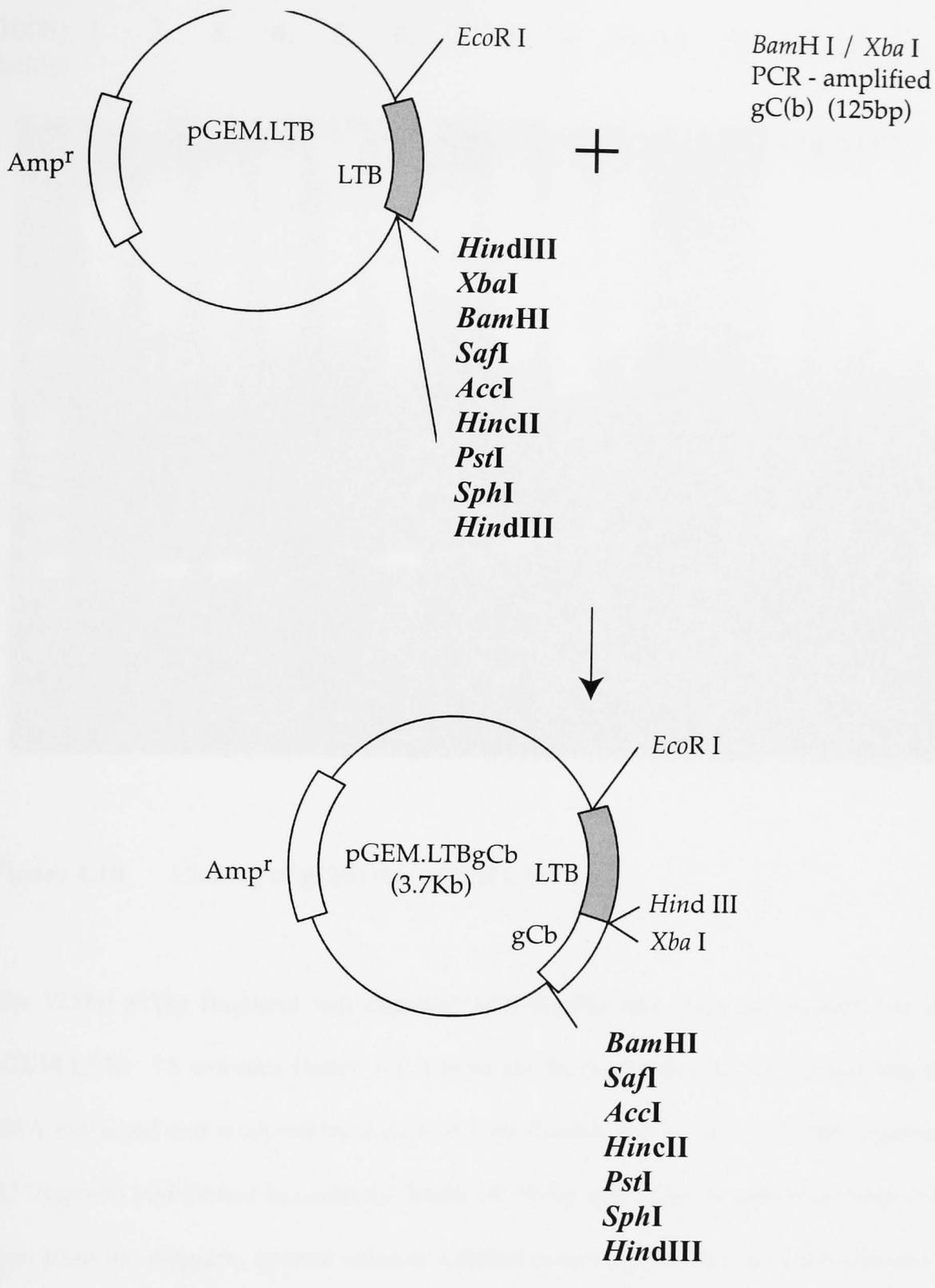


Figure 4.9 Schematic diagram of gC(b) cloning into pGEM.LTB

100bp ladder 1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 100bp ladder

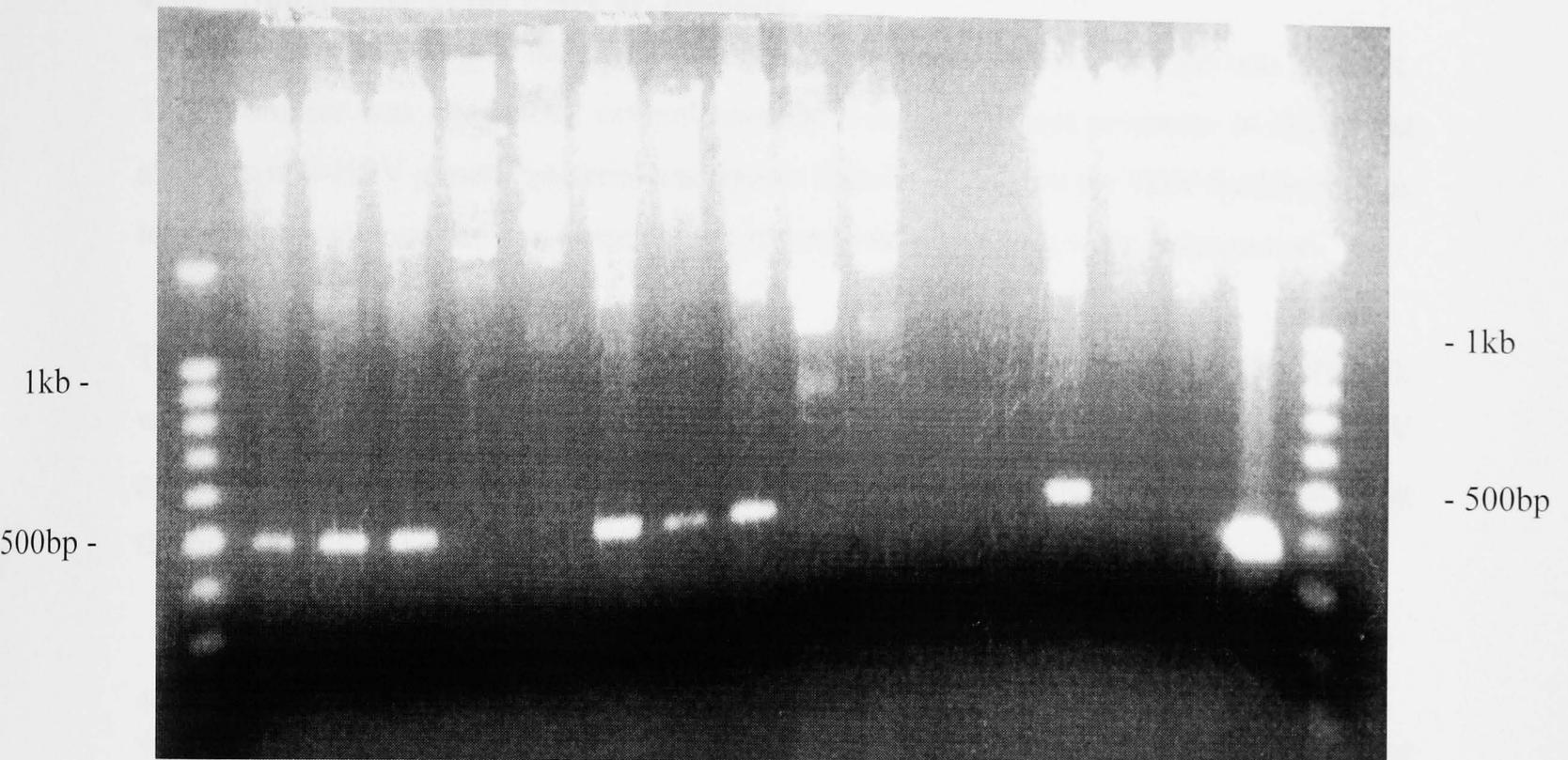


Figure 4.10 Cloning of gC(b) into pGEM.LTB

The 125bp gC(b) fragment was digested with *Bam*HI and *Xba*I and ligated into these sites in pGEM.LTB. 15 colonies (lanes 1-15) from the ligation were grown up and had their plasmid DNA extracted and analysed by digestion with *Bam*HI and *Eco*RI on a 0.8% agarose gel. If the gC fragment had cloned in correctly, bands of 499bp and 3.2kb would result from this digest. As seen from the diagram, several colonies yielded clones. Lane 16 contained digested pGEM.LTB which gives a band of 374bp. Colony number 3 was selected and grown to give plasmid pGEM.LTB.gC(b).

4.5.1 Subcloning of the CMV IE promoter

To drive gene expression, the CMV immediate early promoter was cloned into RL1.del. This promoter was chosen for several reasons: it is an efficient promoter in HSV, and also it is non-HSV genetic material and shows little homology to the HSV backbone-thus lessening the chances of non-homologous recombination, during virus construction.

The CMV immediate early promoter, contained in the plasmid pcDNA3 (Invitrogen), was digested with *NruI* and *XhoI*, removing a 771bp fragment containing the CMV promoter (Figure 4.13). This was cloned into the *NruI* - *XhoI* site of RL1.del, generating the plasmid RL1.dCMV (Figure 4.14).

4.5.2 Subcloning of gC(a)-LTB into RL1.dCMV

To clone the larger gC-LTB fragment into RL1.dCMV, the fragment was PCR-amplified using pGEM.LTBgC(a) as a template. The following primers were used: prLTB, a 25mer, which anneals to the 5' end of *extB* and contains an *XhoI* recognition site (GGGACTCGAGATGAATAAAGTAAAA), and GC5, a 25mer which anneals to the 3' end of the gC gene and contains a *HpaI* recognition site: (GGGTGTAACTTACCGCCGATGACG).

The conditions used were 10 min at 98° C, followed by 30 cycles of 1min at 98° C, 30 sec at 52° C, and 30 sec at 72° C, with final extension at 72° C for 10 min (Figure 4.15).

The 825bp gene fusion was amplified, fractionated on a 1% TAE agarose gel (Figure 4.16) excised using the Nucleon gel extraction kit then digested with *XhoI* and *HpaI* and cloned into these sites within RL1.dCMV, thus creating the 10.1Kb plasmid, RL1.dCMV.LTBgCa (Figure 4.18).

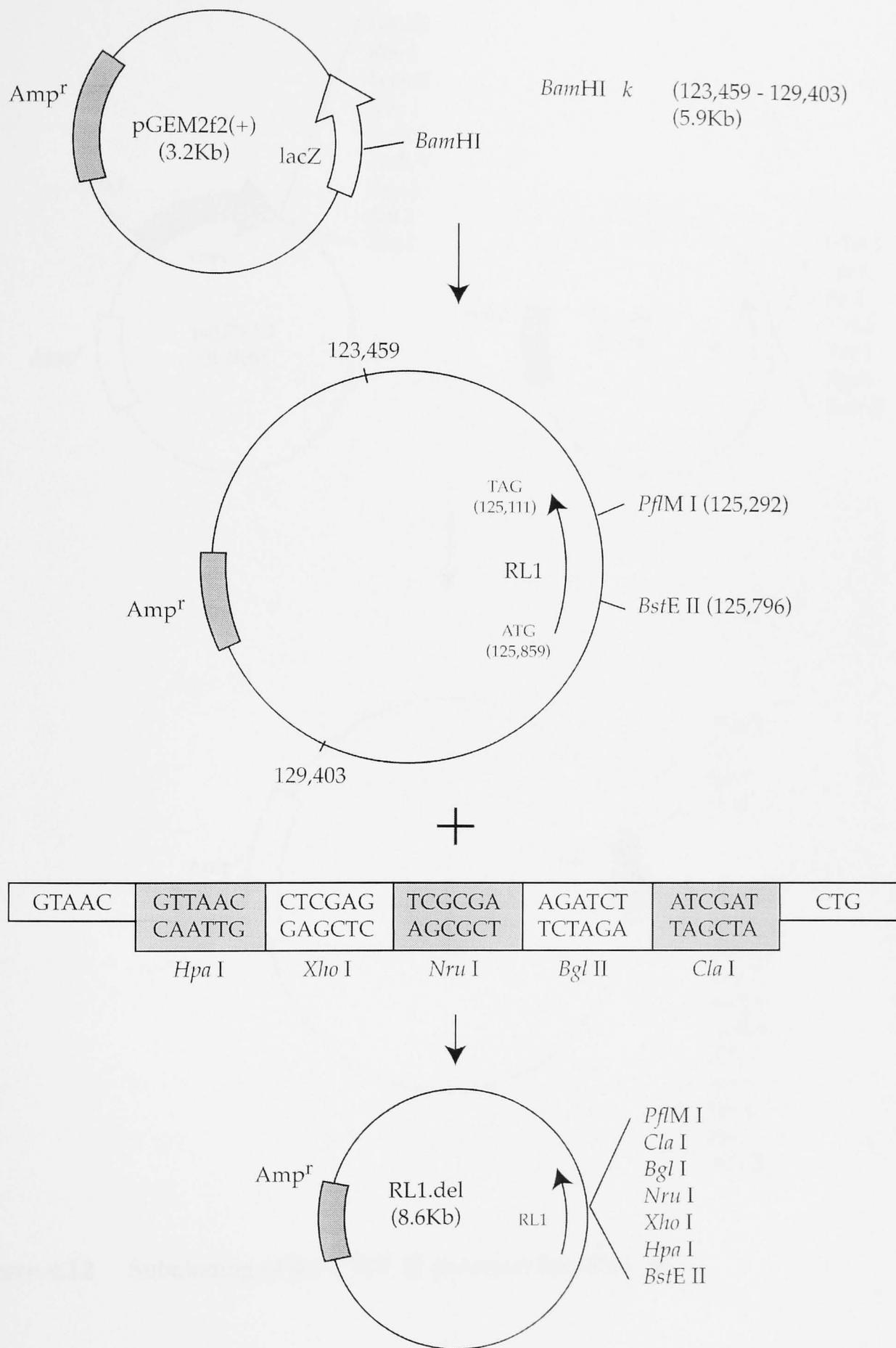


Figure 4.11 Schematic construction of RL1.del

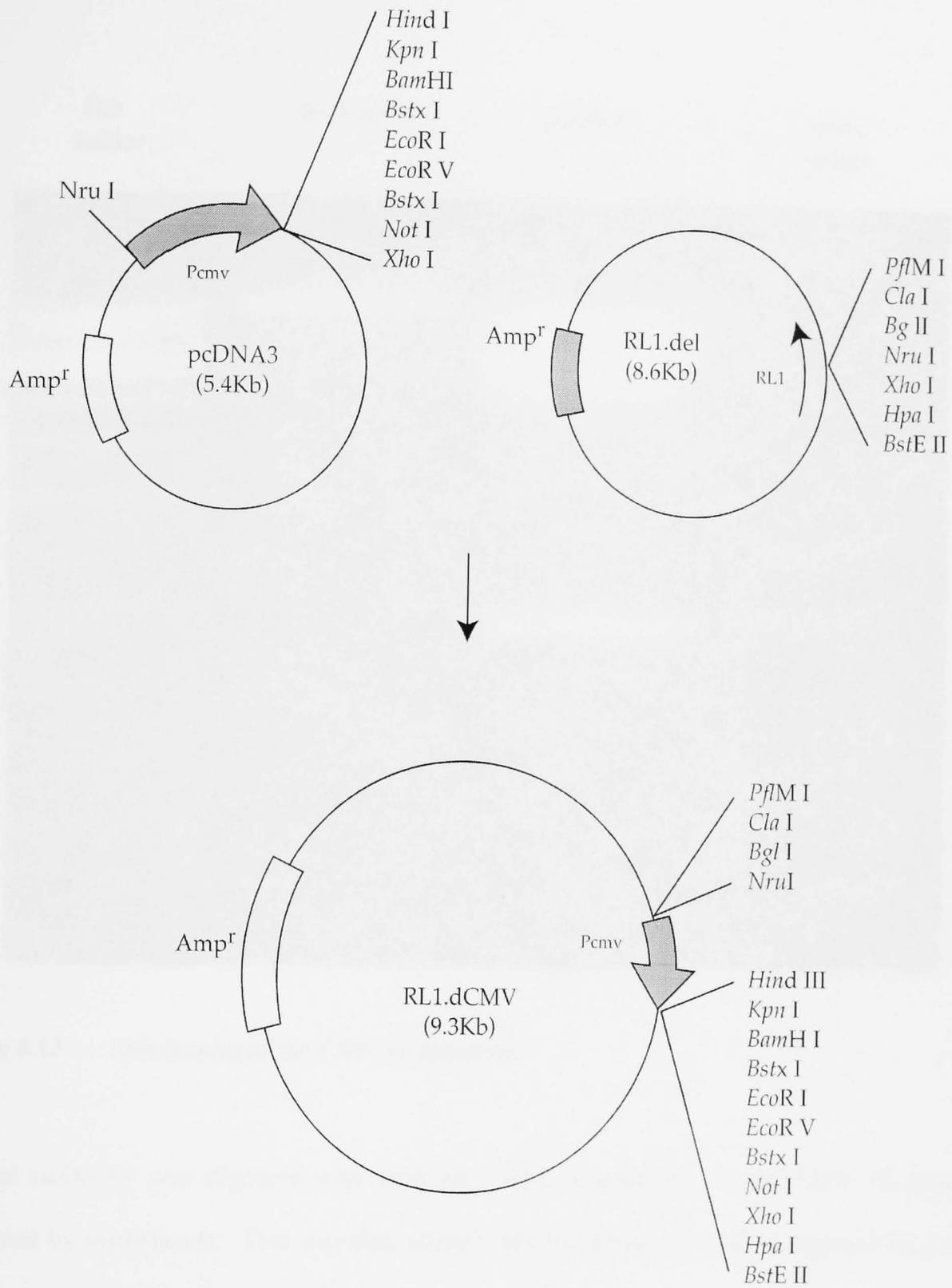


Figure 4.12 Subcloning of the CMV IE promoter into RL1.del

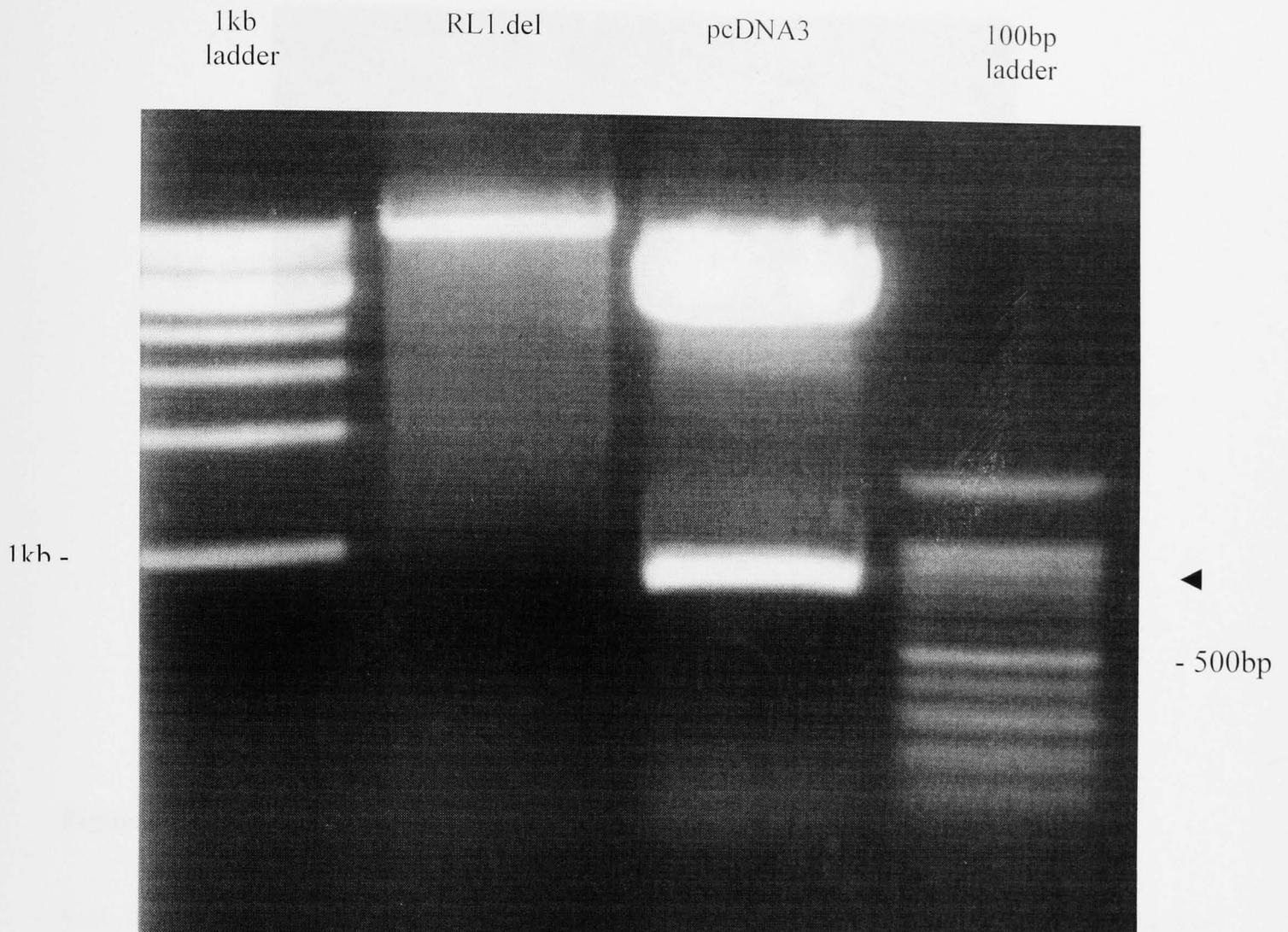


Figure 4.13 Subcloning of the CMV IE promoter

Plasmid pcDNA3 was digested with *NruI* and *XhoI*, cutting the 771bp CMV IE promoter (indicated by arrowhead). This was then cloned into the 8.6kb *NruI/XhoI* digested RL1.del to create the plasmid RL1.dCMV.

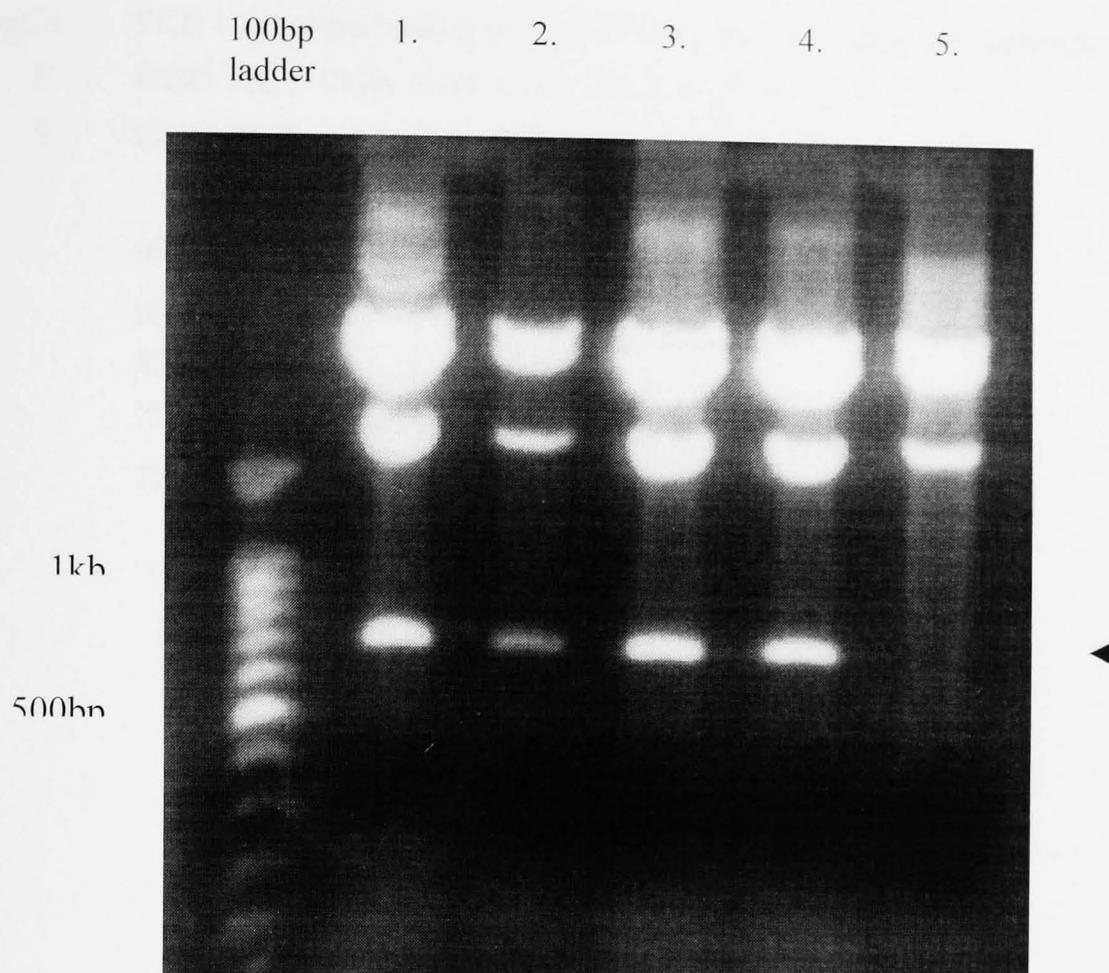


Figure 4.14 Subcloning of the CMV IE promoter into RL1.del

Following the ligation of the CMV IE promoter into the *NruI/XhoI* sites of RL1.del, five colonies were selected (lanes 1-5) and their plasmid DNA extracted and analysed by digestion with *Bam*HI and *Bgl*III on a 1% agarose gel. If the promoter has been cloned, the 771bp promoter will be cleaved from the plasmid, along with 3 other bands of 1.8kb, 3.2kb and 3.5kb, RL1.del only giving the three latter bands. As can be seen from the diagram, the 771bp band (arrowhead) appears in 4 colonies (lanes 1-4). Colony number 2 was chosen and grown up to provide plasmid RL1.dCMV.

LTB-gCa PCR - amplified using pGEM.LTBgCa as a template and primers:

5' GGG ACT CGA GAT GAA TAA AGT AAA A 3'

5' GGG TGT TAA CTT ACC GCC GAT GAC G 3'

98°C - 10 mins

98°C - 1 min

52°C - 30 secs

72°C - 30 secs

30 cycles

72°C - 10 mins

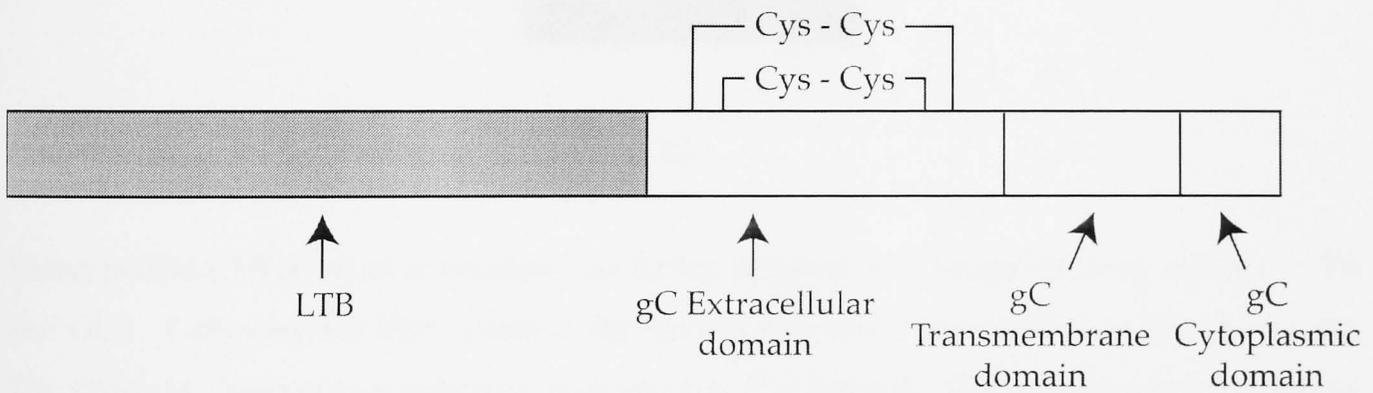
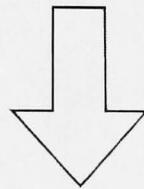


Figure 4.15 Schematic diagram of LTB-gC(a) PCR

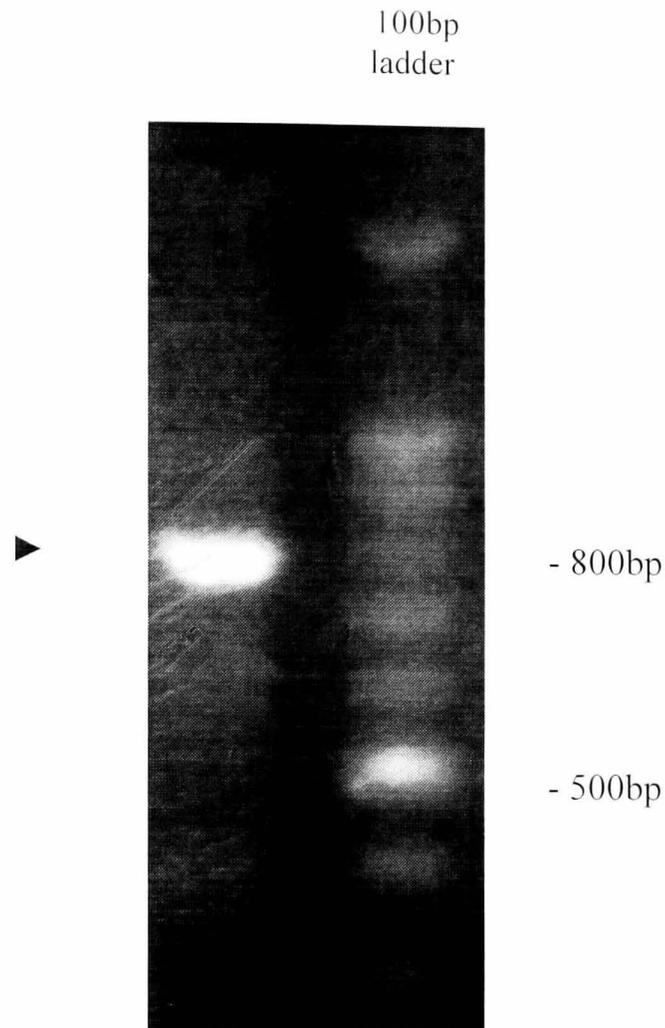


Figure 4.16 PCR-amplification of LTB-gC(a)

Using pGEM.LTBgC(a) as a template, the 825bp fragment was amplified using primers pLTB and GC5. Following the PCR reaction, the reaction mix was fractionated on a 1% agarose gel. The 825bp gC fragment (as indicated by arrow) was digested with *Xho*I and *Hpa*I and then cloned into these sites in plasmid RL1.dCMV.

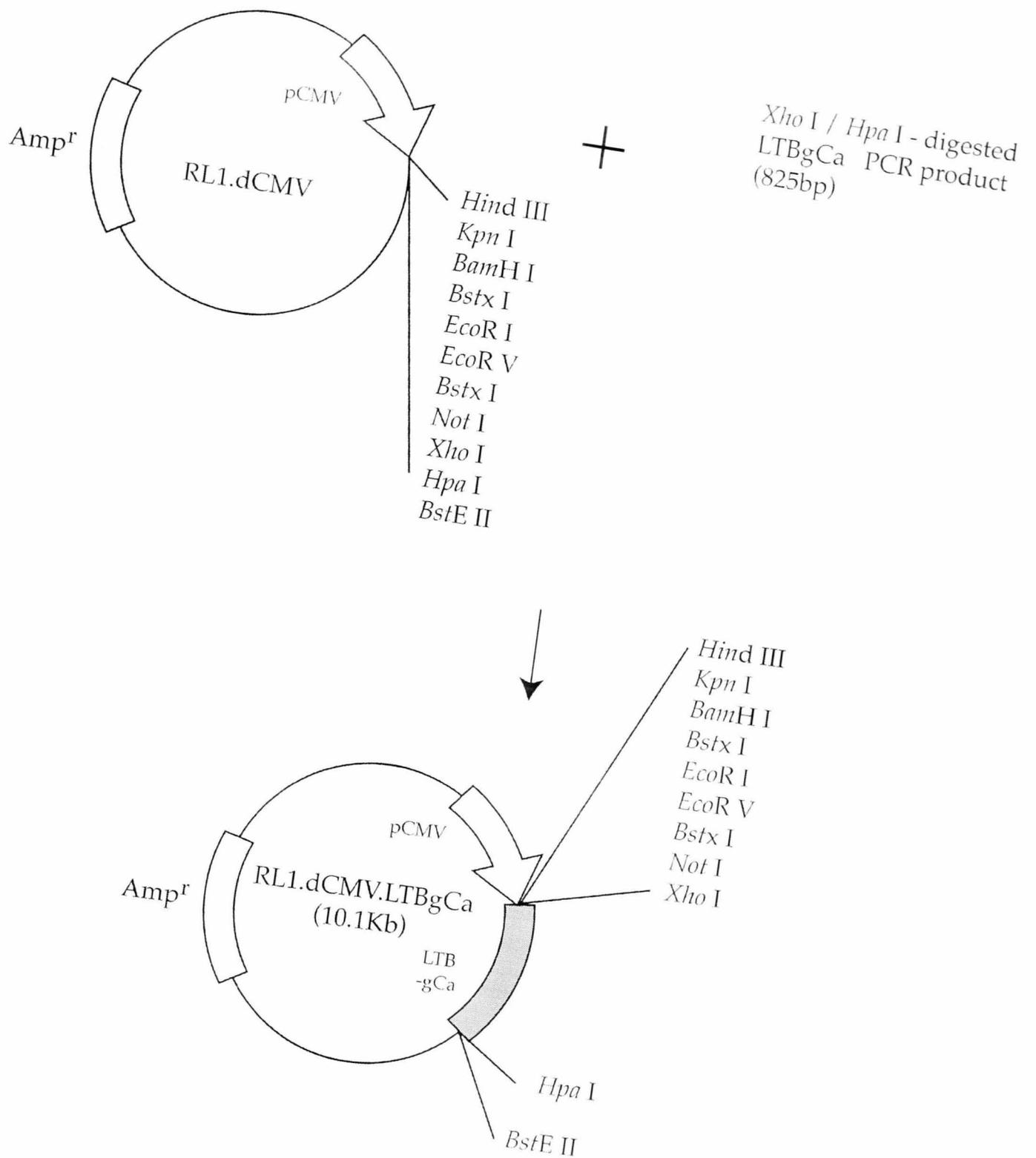


Figure 4.17 Schematic diagram of LTBgC(a) cloning into RL1.dCMV

100bp ladder 1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 100bp ladder

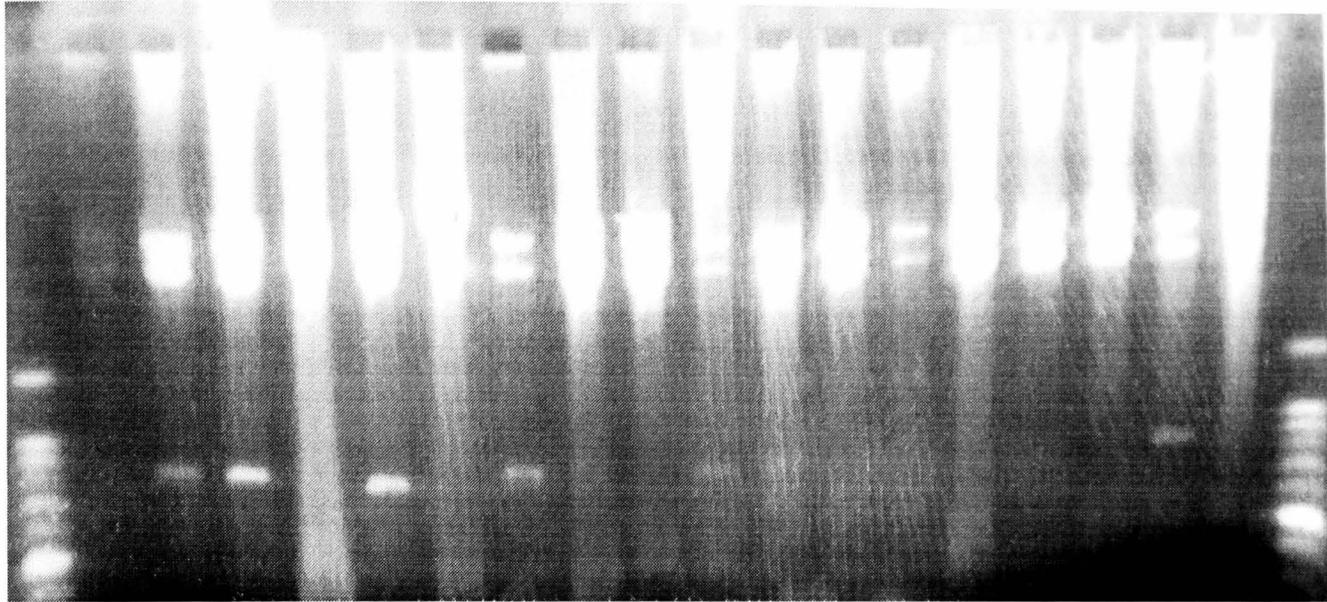


Figure 4.18 Cloning of LTBgC(a) into RL1.dCMV

Following ligation of the *XhoI/HpaI* digested LTBgC(a) PCR fragment to RL1.dCMV, 18 colonies (labelled 1-18) were selected and their plasmid DNA extracted and analysed by digestion with *EcoRI/HpaI* on a 0.8% agarose gel. If the fragment had been cloned, digestion would yield bands of 825bp (the inserted fragment), 3.7kb and 5.5kb. RL1.dCMV yields the latter two bands. As seen from the diagram several colonies contained the cloned fragment. Number 3 was selected and grown up to give plasmid RL1.dCMV.LTBgC(a).

LTB-gCb PCR - amplified using pGEM.LTBgCb as a template and primers:

5' GGG ACT CGA GAT GAA TAA AGT AAA A 3'

5' GGG TGT TAA CTT ACC GCC GAT GAC G 3'

98°C - 10 mins

98°C - 1 min

52°C - 30 secs

72°C - 30 secs } 30 cycles

72°C - 10 mins

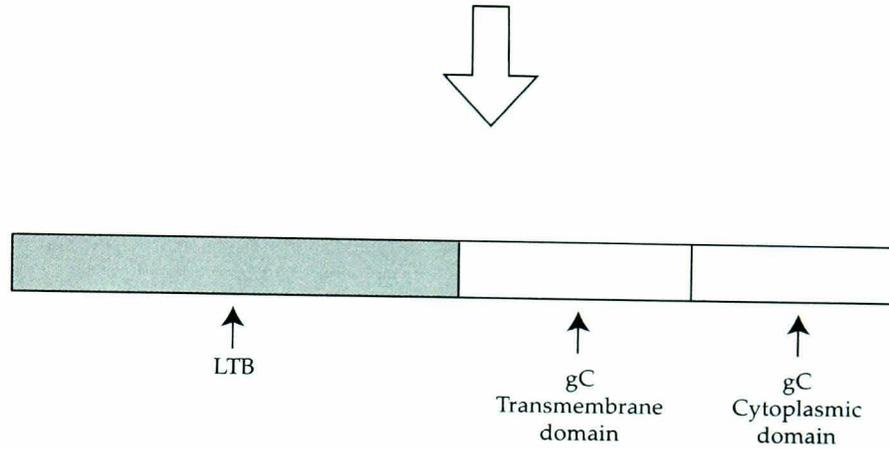


Figure 4.19 Schematic drawing of LTB-gC(b) PCR

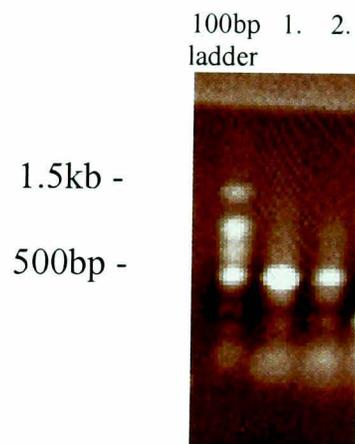


Figure 4.20 PCR-amplification of LTB-gC(b)

Using pGEM.LTBgC(b) as a template, the 525bp gene fusion was amplified using primers pLTB and GC5. Two concentrations of template and primer were used and the PCR reaction mix was fractionated on a 1% agarose gel. The 525bp fragment (as indicated by arrow) was digested with *Xho*I and *Hpa*I and cloned into these sites in RL1.dCMV.

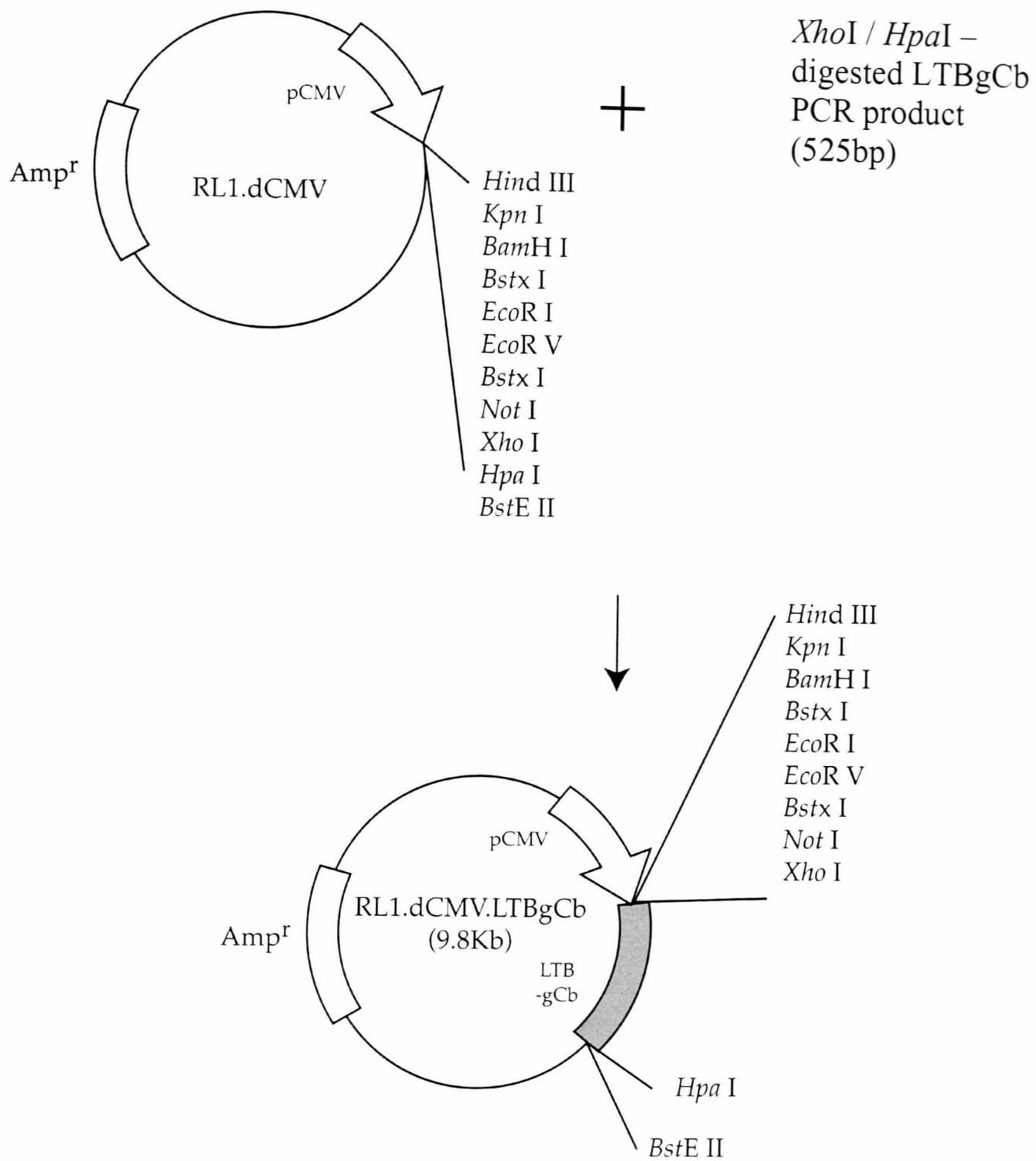


Figure 4.21 Schematic diagram of LTBgC(b) cloning into RL1.dCMV

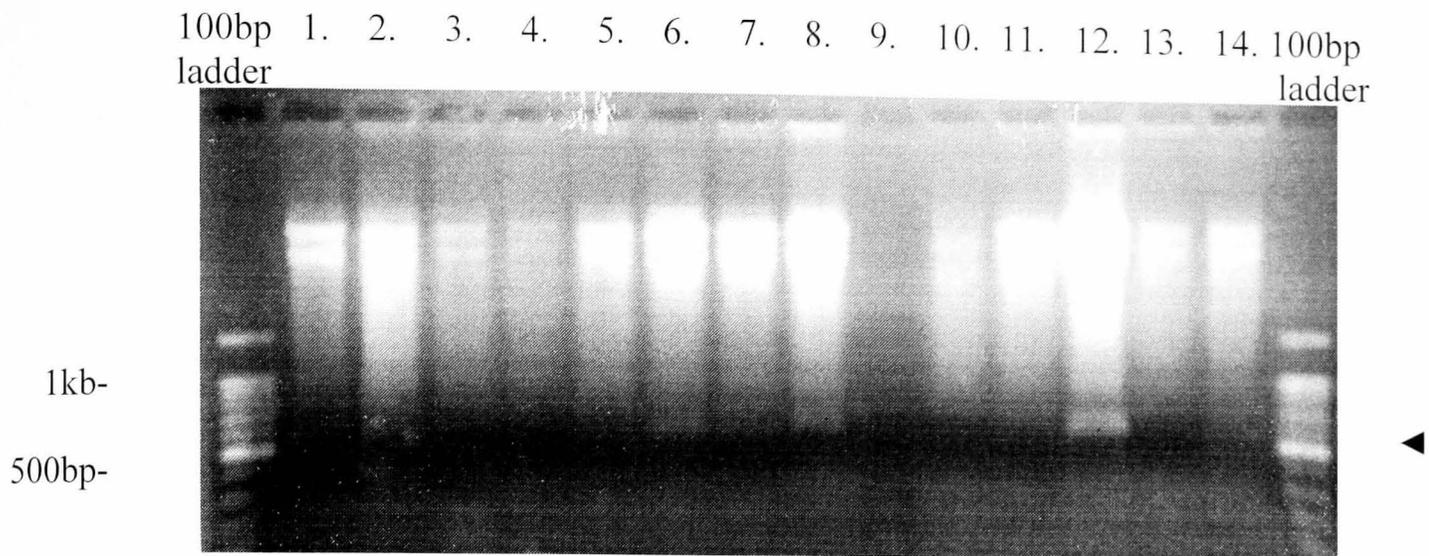


Figure 4.22 Cloning of LTB-gC(b) into RL1.dCMV

Following the ligation of *XhoI/HpaI*-digested LTB-gC(b) to RL1.dCMV, 14 colonies (labelled 1-14) were picked. They had their plasmid DNA extracted and analysed by digestion with *EcoRI/HpaI*, on a 1% agarose gel. If the fragment had been cloned, three bands would be formed by the digestion: 525bp (LTB-gC(b) fragment), 3.7kb and 5.5kb, otherwise, just the two latter bands would be formed. As can be seen from the diagram, colony 12 gave the 525bp band (indicated by arrowhead). This was then selected and grown up to provide plasmid pGEM.LTB-gC(b).

4.5.3 Subcloning of gC(b)-LTB into RL1.dCMV

To clone the smaller gC-LTB fragment into RL1.dCMV, the fragment was PCR-amplified using pGEM.LTB.gC(b) as a template. The following primers were used: prLTB, a 25mer, which anneals to the 5' end of *extB* and contains an *Xho*I recognition site (GGGACTCGAGATGAATAAAGTAAAA), and GC5, a 25mer which anneals to the 3' end of the gC gene and contains a *Hpa*I recognition site: (GGGTGTTAACTTACCGCCGATGACG).

The conditions used were 10 min at 98° C, followed by 30 cycles of 1min at 98° C, 30 sec at 52° C, and 30 sec at 72° C, with final extension at 72° C for 10 min (Figure 4.19).

The 525bp gene fusion was amplified, fractionated on a 1% TAE agarose gel (Figure 4.20), excised using the Nucleon gel extraction kit then digested with *Xho*I and *Hpa*I and cloned into these sites within RL1.dCMV, thus creating the 9.8Kb plasmid, RL1.dCMV.LTBgCb (Figure 4.21).

4.5.4 Subcloning of a PolyA site into the expression plasmids

As both expression plasmids were missing polyA sequences, the HSV-2 IE5 polyadenylation sequence was subcloned from plasmid pFJ14 (A.R.MacLean) into both RL1.dCMV.LTBgCa and RL1.dCMV.LTBgCb. pFJ14 was digested with *Bam*HI and *Hin*dIII, excising the 114bp polyA sequence (Figure 4.23). This was Klenow-treated to blunt-end the fragment, which was then cloned into the *Hpa*I site of the expression plasmids RL1.dCMV.LTBgCa, and RL1.dCMV.LTBgCb to create plasmids pRFa and pRFb respectively (Figure 4.24, Figure 4.26). To determine if the inserts were in the correct orientation, clones were digested with *Eco*RI and *Pst*I. A *Pst*I site exists within the polyA sequence. For pRFa, a band of 785bp signified a clone with the polyA site in the correct orientation (Figure 4.25) and for pRFb a band of 485bp signified the polyA sequence had inserted correctly (Figure 4.27).

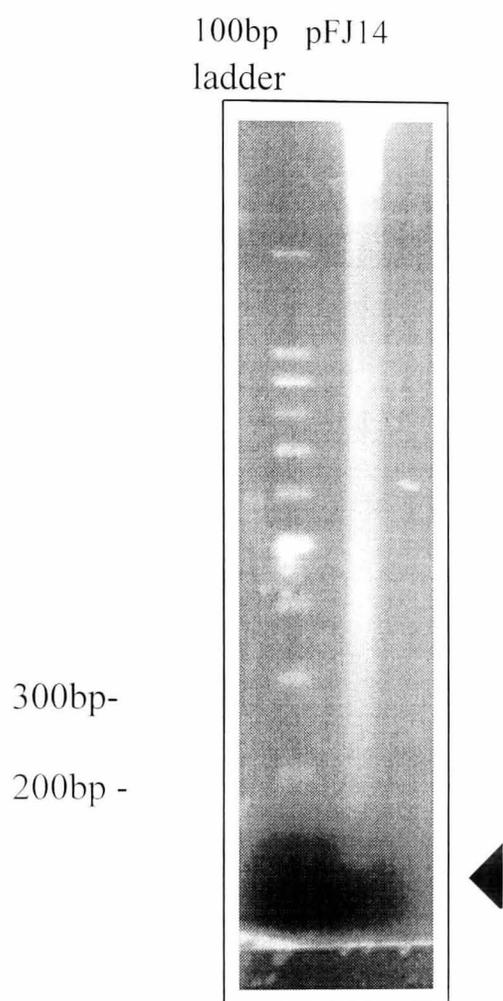
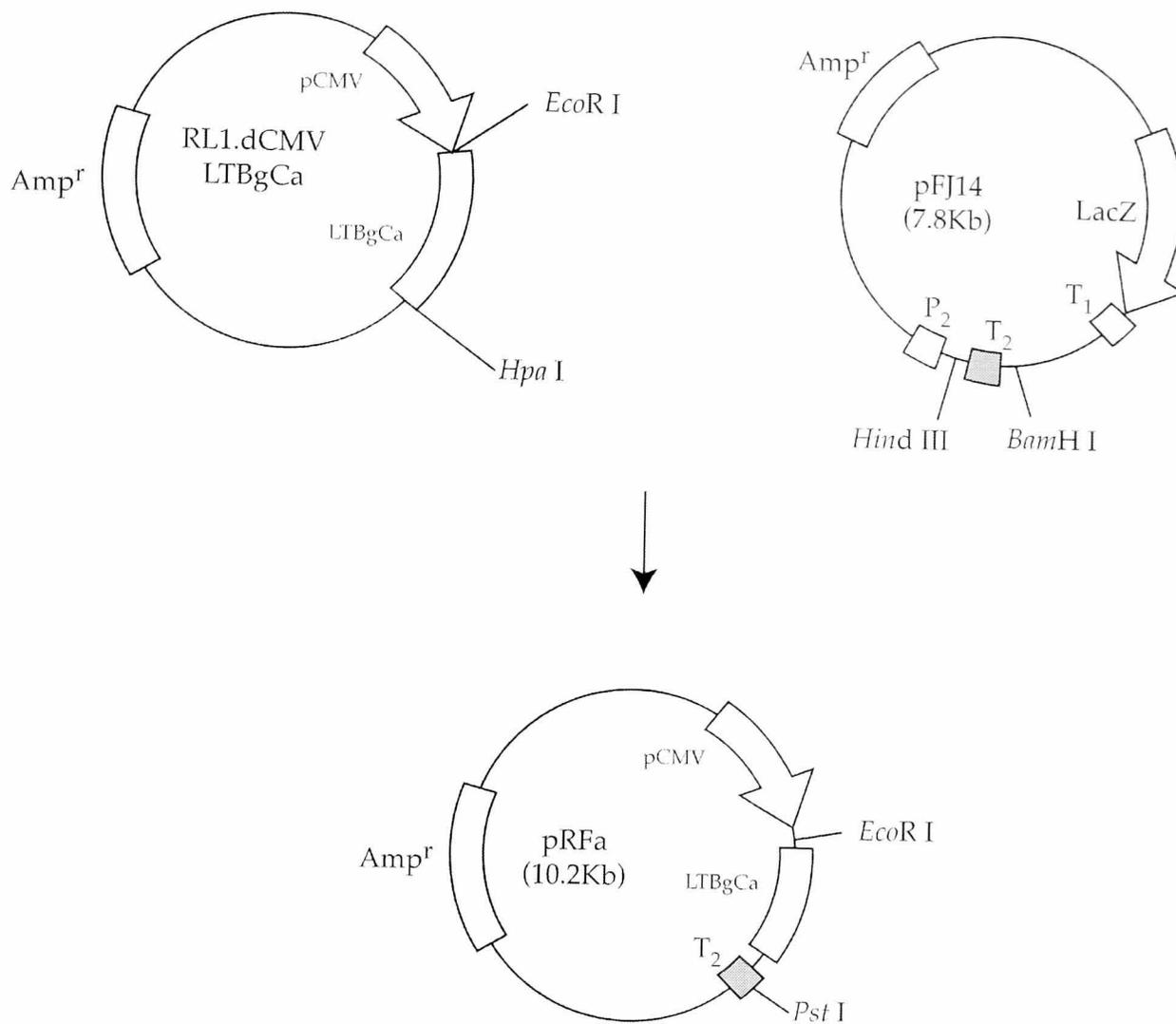


Figure 4.23 Subcloning of the HSV-2 IE5 polyadenylation sequence

Plasmid pFJ14 was digested with *Bam*HI and *Hin*dIII, excising the 114bp HSV-2 IE5 polyadenylation sequence (faint band above dye front, indicated by arrowhead). This was then treated with Klenow to blunt-end the fragment, before cloning into the *Hpa*I site in plasmids RL1.dCMV.LTB-gC(a) and RL1.dCMV.LTB-gC(b).



LEGEND:

- T1: SV40 early polyadenylation sequence
- T2: HSV-1 gD promoter
- T2: HSV-2 IE5 polyadenylation sequence

Figure 4.24 Schematic diagram of subcloning of PolyA into RL1.dCMV.LTBgCa

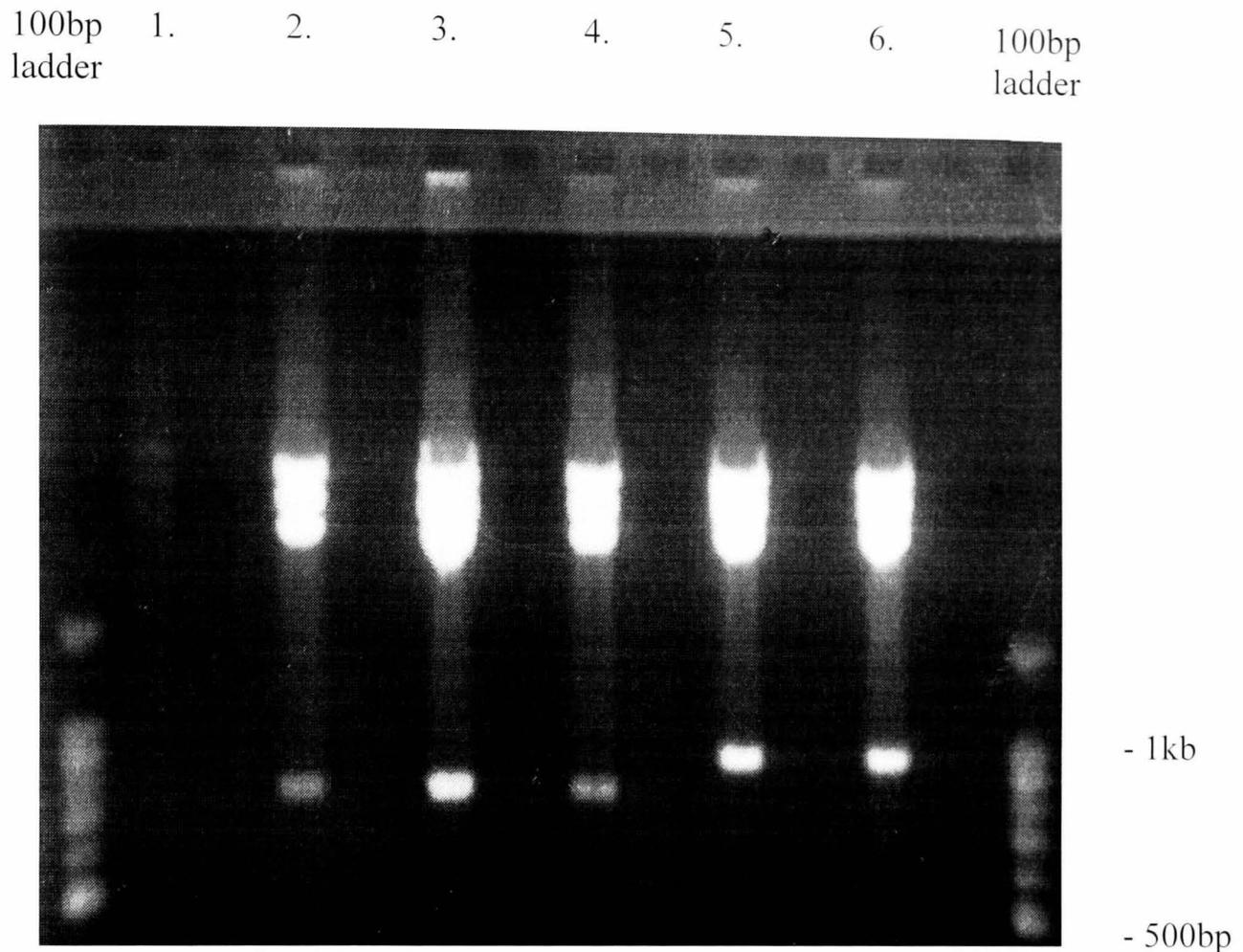
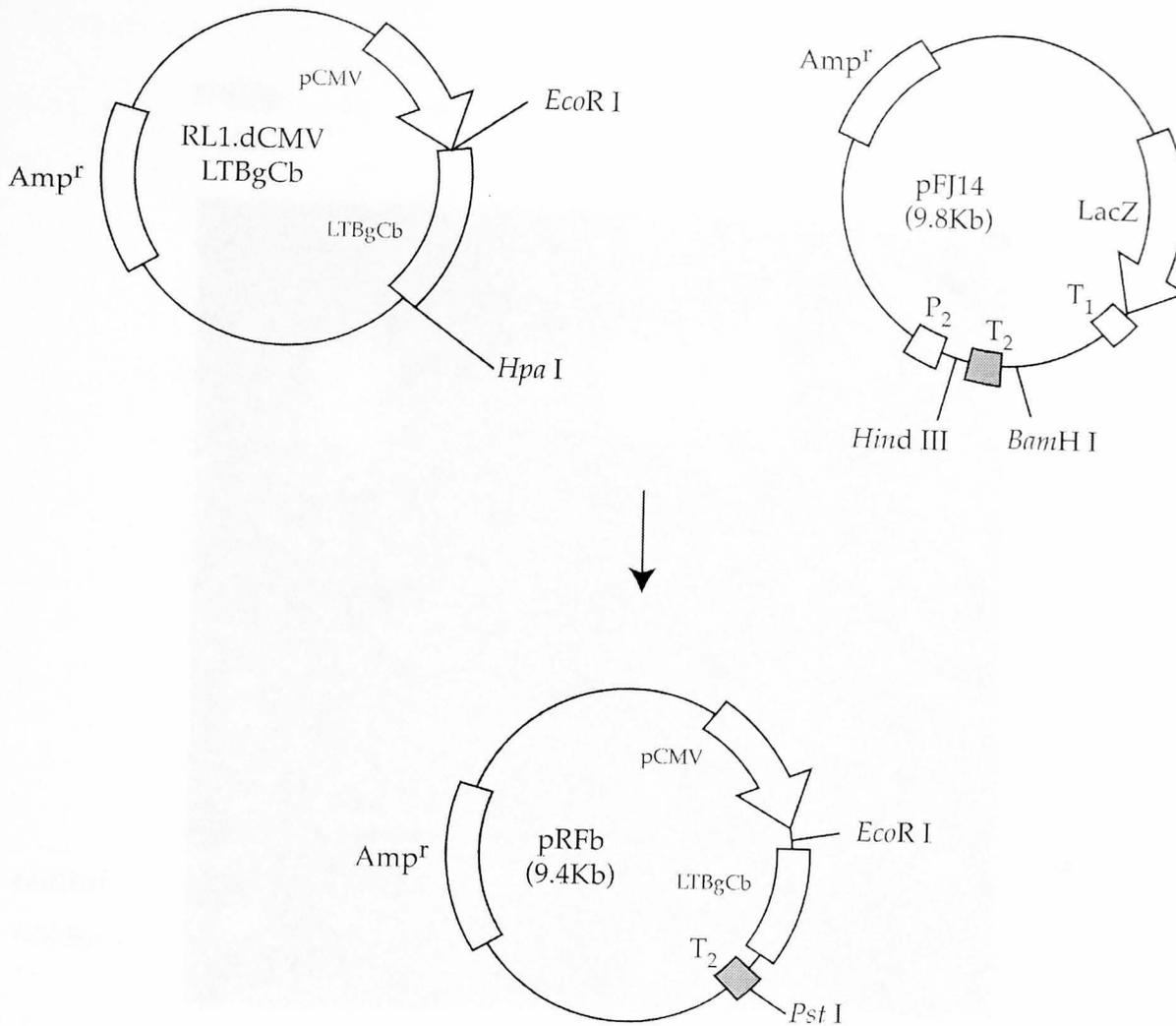


Figure 4.25 Cloning of PolyA into RL1.dCMV.LTB-gC(a)

Following ligation of the 119bp CMV IE polyadenylation sequence into RL1.dCMV.LTB-gC(a) 6 colonies were selected (lanes 1-6), their plasmid DNA extracted, digested with *EcoRI* and *PstI*, and ran on a 1% agarose gel. The CMV IE polyadenylation sequence contains a *PstI* site starting at base-pair position 4. If the PolyA sequence inserted in the correct orientation, this digest will cut out a fragment of 804bp(indicated by arrow). If however, incorrect orientation has occurred, then a band of 919bp will be cut (three other bands of 2.5kb, 3.2kb, and 3.7kb are also cut). As can be seen from the diagram, colonies 2-4 have the PolyA sequence in the correct orientation, whereas numbers 5 and 6 have the sequence cloned in an inverted position. Number 3 was chosen and grown up to provide plasmid pRFa.



LEGEND:

- T1: SV40 early polyadenylation sequence
- T2: HSV-1 gD promoter
- T2: HSV-2 IE5 polyadenylation sequence

Figure 4.26 Schematic diagram of subcloning of PolyA into RL1.dCMV.LTBgCb

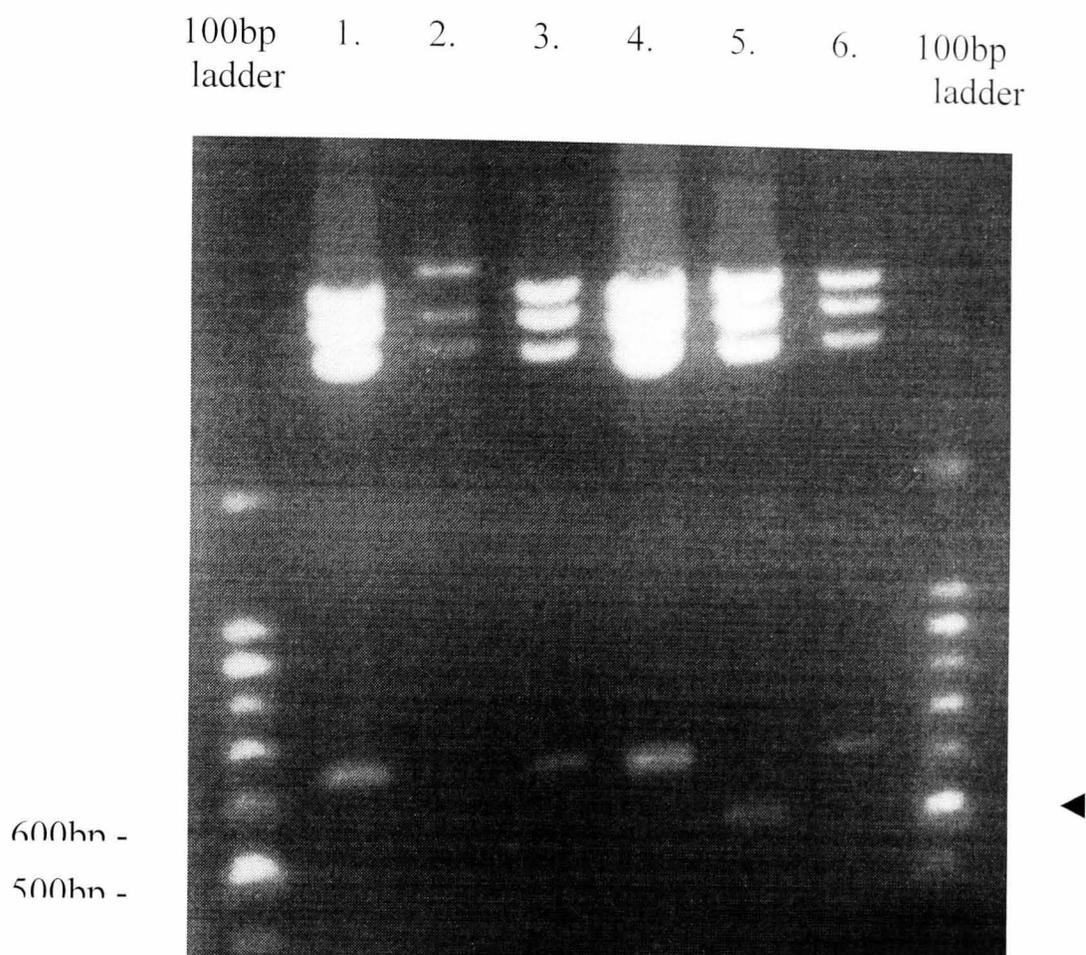


Figure 4.27 Cloning of PolyA into RL1.dCMV.LTB-gC(b)

Following ligation of the 119bp CMV IE polyadenylation sequence into RL1.dCMV.LTB-gC(b) 6 colonies were selected (lanes 1-6), their plasmid DNA extracted, digested with *EcoRI* and *PstI*, and ran on a 1% agarose gel. The CMV IE polyadenylation sequence contains a *PstI* site starting at base-pair position 4. If the PolyA sequence inserted in the correct orientation, this digest will cut out a fragment of 504bp. If however, incorrect orientation has occurred then a band of 619bp will be cut (three other bands of 2.5kb, 3.2kb, and 3.7kb are also cut). As can be seen from the diagram, colony 5 has the PolyA sequence in the correct orientation (faint band, indicated by arrowhead), whereas the other colonies have the sequence cloned in an inverted position. Number 5 was chosen and grown up to provide plasmid pRFa.

4.5.5 Sequencing of pRFa and pRF

Following cloning of the polyA sequence the plasmids were sequenced by DNAshef, Edinburgh. The sequences were returned as follows:

pRFa:

```
CTC GAG ATG AAT AAA GTA AAA TTT TAT GTT TTA TTT ACG GCG TTA
CTA TCC TCT CTA TGT GCA CAC GGA GCT CCT CAG TCT ATT ACA GAA
CTA TGT TCG GAA TAT CAC AAC ACA CAA ATA TAT ACG ATA AAT GAC
AAG ATA CTA TCA TAT ACG GAA TCG ATG GCA GGC AAA AGA GAA ATG
GTT ATC ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC
CCG GGC AGT CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG
ATG AAG GAC ACA TTA AGA ATC ACA TAT CTG ACC GAG ACC AAA ATT
GAT AAA TTA TGT GTA TGG AAT AAT AAA ACC CCC AAT TCA ATT GCG
GCA ATC AGT ATG GAA AAG CTT ATG GAA TTT GGG GTC CGC ATT GTG
GTC TGC ACG GCC GGC TGC GTC CCC GAG GGC GTG ACG TTT GCC TGG
TTC CTG GGG GAC GAC CCC TCA CCG GCG GCT AAG TCG GCC GTT ACG
GCC CAG GAG TCG TGC GAC CAC CCC GGG CTG GCT ACG GTC CGG TCC
ACC CTG CCC ATT TCG TAC GAC TAC AGC GAG TAC ATC TGT CGG TTG
ACC GGA TAT CCG GCC GGG ATT CCC GTT CTA GAA CAC CAC GGC AGT
CAC CAG CCC CCA CCC AGG GAC CCC ACC GAG CGG CAG GTG ATC GAG
GCG ATC GAG TGG GTG GGG ATT GGA ATC GGG GTC CTC GCG GCG GGG
GTC CTG GTC GTA ACG GCA ATC GTG TAC GTC GTC CGC ACA TCA CAG
TCG CGG CAG CGT CAT CGG CGG TAA GTT AAC
```

Sequencing showed the gene fusion to be in-frame. The initiating codon of LTB (ATG) is shown in bold. Also shown in bold is the *Hind*III site at the LTB-gC gene fusion junction.

This sequence was then translated electronically using the VectorNTI software package:

Translation of LTBgCa ORF from base pairs 7 to 786:

```
mnkvkfyvlfallsslcahgapqsitelcseyhntqiytindkilsytesmagkremviitfksgatfqvevpgsqhidsqkk
aiermkdtrityltetkidklcvwnnktpnssiaaismeklmefgvriivvctagcvpegvtfawflgddpspaaksavtaqes
cdhpglatvrstlpisydyseyicrltgypagipvlehghshqppprdpterqvieaiewvgigigvlaagvlvvtaiyvvr
sqsrqrhrr
```

pRFb:

CTC GAG **ATG** AAT AAA GTA AAA TTT TAT GTT TTA TTT ACG GCG TTA
CTA TCC TCT CTA TGT GCA CAC GGA GCT CCT CAG TCT ATT ACA GAA
CTA TGT TCG GAA TAT CAC AAC ACA CAA ATA TAT ACG ATA AAT GAC
AAG ATA CTA TCA TAT ACG GAA TCG ATG GCA GGC AAA AGA GAA ATG
GTT ATC ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC
CCG GGC AGT CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG
ATG AAG GAC ACA TTA AGA ATC ACA TAT CTG ACC GAG ACC AAA ATT
GAT AAA TTA TGT GTA TGG AAT AAT AAA ACC CCC AAT TCA ATT GCG
GCA ATC AGT ATG GAA **AAG CTT TCT AGA** TGG GTG GGG ATT GGA ATC
GGG GTC CTC GCG GCG GGG GTC CTG GTC GTA ACG GCA ATC GTG TAC
GTC GTC CGC ACA TCA CAG TCG CGG CAG CGT CAT CGG CGG TAA GTT
AAC

Sequencing showed the gene fusion to be in-frame. The initiating codon of LTB (ATG) is shown in bold. Also shown in bold is the *HindIII* and *XbaI* sites at the LTB-gC gene fusion junction.

This sequence was then translated electronically using the VectorNTI software package:

Translation of LTBgCb ORF from base pairs 7 to 486:

mnkvkfyvlf tallsslcahgapqsitelcseyhntqiytindkilsytesmagkremviitfksgatfqvevpgsqhidsqkk
aiermkdtrityltetkidklcvwnnktpnsiaaismeklsrwvgigigvlaagvlvvtaiyvvrtsqsrqrhrr

Sequence Alignment LTB and pRFa/b

pRFa: **mnkvkfyvlf tallsslcahgapqsitelcseyhntqiytindkilsyte**

pRFb: **mnkvkfyvlf tallsslcahgapqsitelcseyhntqiytindkilsyte**

LTB: **mnkvkcyvlf tallsslcaygapqsitelcseyrntqiytindkilsyte**

pRFb: **smagkremviitfksgatfqvevpgsqhidsqkkaiermkdtritylte**

pRFb: **smagkremviitfksgatfqvevpgsqhidsqkkaiermkdtritylte**

LTB: **smagkremviitfksgatfqvevpgsqhidsqkkaiermkdtritylte**

pRFa: **tkidklcvwnnkt****pn**siaaism**ek**lmefgvrivvctagcvpegvtfawfl
 pRFb: **tkidklcvwnnkt****pn**siaaism**ek**lsrwvgigigvlaagvlvvtaiivyv
 LTB: **tkidklcvwnnkt****pn**siaaism**en**

pRFa: gddpspaaksavtaqescdhp~~glatvrst~~lpisydyseyicrltgypa
 pRFb: rtsqsrqrhr

pRFa: gipvlehhgshqpppr~~dpter~~qvieaiewvgigigvlaagvlvvtaiivyv

pRFa: vrtsqsrqrhrr

The sequences were aligned using the VectorNTI software. Both pRFa and pRFb showed homology to the *extB* gene, as indicated by bold lettering. Some sequence differences were seen at amino acids -16, -2, 13 and 103, as indicated by red lettering. These are due to the following sequence differences:

LTB: -16 (TTT)	pRFa: (TGT)	pRFa: (TGT)
-2 (CAC)	(TAC)	(TAC)
13 (CAC)	(CGC)	(CGC)
103 (AAC)	(AAG)	(AAG)

For the first three sequences, the differences are due to the origin of the LTB gene sequence. For the purpose of gene alignment the LTB sequence was taken from NCBI (accession number S60731). These nucleotide differences at positions -16, -2 and 13 have been reported by Leong *et al.*, (1985), as sequencing differences between LTB subunits of different strains i.e. LTB from an enterotoxigenic *E. coli* strain infectious for humans (Leong *et al.*, (1985)) and LTB from a porcine *E. coli* isolate (Dallas and Falkow, 1980). The gene sequence for pRFa and pRFb is in 100% agreement with the sequence of *extB* published by Leong *et al.*, (1985). The final sequence difference (AAG instead of AAC) is due to the *HindIII* site that was inserted at the end of the LTB gene.

Sequence Alignment HSV-1 gC and pRFa/b

gC: mapgrvglavvlwglwlgagvaggsetastgptitagavnaseaptsg
gC: spgsaaspevtptstpnnpnvtqnkttptepaspptpkptstpksppts
gC: tdpkpknnttpaksgprtkppgpvwcdrrdplarygsrvqircrfmst
gC: rmefrlqiwrysmgpppiapapdleevltnitappgllvydsapnlt

pRFa: mnk
gC: phvlwaegagpgadpplysvtgpplptqrliigevtpatqgmyylawgrmd

pRFa: vkfyvlfallsslcahgapqsitelcseyhntqiytindkilsytesma
gC: spheygtwvrvrmfrppsltlqphavmegqpfkatctaaayyprmpvefd

pRFa: gkremviitfksgatfqvevpgsqhidsqkkaiermkdtrityltetki
gC: drqvfnpqqidqtthehpdgfttvstvtseavggqpprtftcqmthrd
pRFb: mnkvkf

pRFa: dklcvwnnktpnsiaaismekl**mefgvrivvctagecvpegvtfawflgdd**
gC: svtfrrnatglalvlprptit**mefgvrivvctagecvpegvtfawflgdd**
pRFb: yvlfallsslcahgapqsitelcseyhntqiytindkilsytesmagkr

pRFa: **pspaaksavtaqescdhpplatvrstlpisydyseyicrltgypagipvl**
gC: **pspaaksavtaqescdhpplatvrstlpisydyseyicwltgypagipvl**
pRFb: emviitfksgatfqvevpgsqhidsqkkaiermkdtrityltetkidkl

pRFa: **ehhghqppprdpterqvieaiewvgigigvlaagvlvtaivyvvrtsqsr**
gC: **ehhghqppprdpterqvieaiewvgigigvlaagvlvtaivyvvrtsqsr**
pRFb: cvwnnktpnsiaaismeklsrwwgigigvlaagvlvtaivyvvrtsqsr

pRFa: **qrhrr**
gC: **qrhrr**
pRFb: **qrhrr**

The sequences were aligned using the VectorNTI software. Both pRFa and pRFb showed homology to HSV-1 gC, as indicated by bold lettering (pRFa showed homology to amino acids 377-511 of gC; pRFb showed homology to amino acids 478-511 of gC).

4.6 Co-transfection of pRFa/pRFb with HSV-1 gC-ve DNA

4.6.1 Generation of RFa and RFb

Plasmids pRFa and pRFb were digested with *Xmn*I and co-transfected with gC-ve (lacZ) DNA onto BHK cells, as described in section 3.23, to generate new recombinant viruses RFa and RFb, respectively. The transfection plates were incubated until cpe was complete. The infected cells were centrifuged, virus released by sonication, and titrated on BHK monolayers. Discreet plaques were picked and used to infect BHK cells in 24-well linbro dishes. Two days post-infection, the medium was removed, and stored, from the linbro wells, and the viral DNA extracted (section 3.26). The DNA was digested with *Eco*RI and Southern blotted (section 3.28) using a ³²P-RL1.del probe.

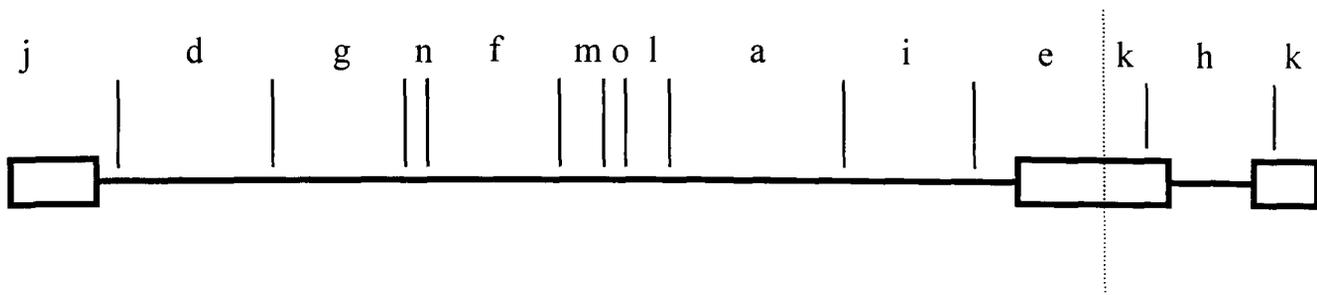
Both RFa and RFb have an additional *Eco*RI site in the region recognised by the RL1.del probe (Figure 4.29), thus on Southern blotting the recombinant viruses generate a different pattern of bands (Figure 4.30).

When a recombinant viral plaque was isolated it was then taken through a further two rounds of plaque purification (section 3.25), with Southern blotting carried out at each step to ensure the purity of the virus.

By the end of the third round of plaque purification, recombinant virus RFb, was completely pure as determined by the presence of a novel 6.8kb band (Figure 4.31). The virus was shown to be free of contaminating background virus by the absence of a 21kb band seen only in the parental virus. A ten-roller bottle stock was grown on BHK cells to generate a stock of purified virus (section 3.10).

Following co-transfection of pRFa and gC-ve (LacZ) DNA, a positive recombinant virus was detected by Southern blotting as detected by the presence of the indicative 7.1kb band. Attempts to purify the virus failed, with the recombinant virus lost, as determined by Southern blotting, between the 1st and 2nd round of plaque purification. Further recombinant viruses failed to be completely purified. Southern blotting of the partially purified virus shows the 7.1kb band, indicative of the new recombinant virus, but also contamination with background virus, as the 21kb band was present (Figure 4.31).

a.



b.

<u>EcoRI Fragments</u>	<u>Molecular Size (bp)</u>
a	21835
b	21439
c	17753
d	16747
e	16277
f	16232
g	16135
h	15160
i	13344
j	12591
k	5566
l	5219
m	4073
n	2416
o	1491

Figure 4.28 Schematic diagram of *EcoRI*-digested HSV-1

- a. A schematic representation of the twelve *EcoRI* sites within the HSV-1 genome
- b. A reference to the molecular weights of each of the fragments. Fragment b is formed by bands e + k, while band c is formed by bands j + k

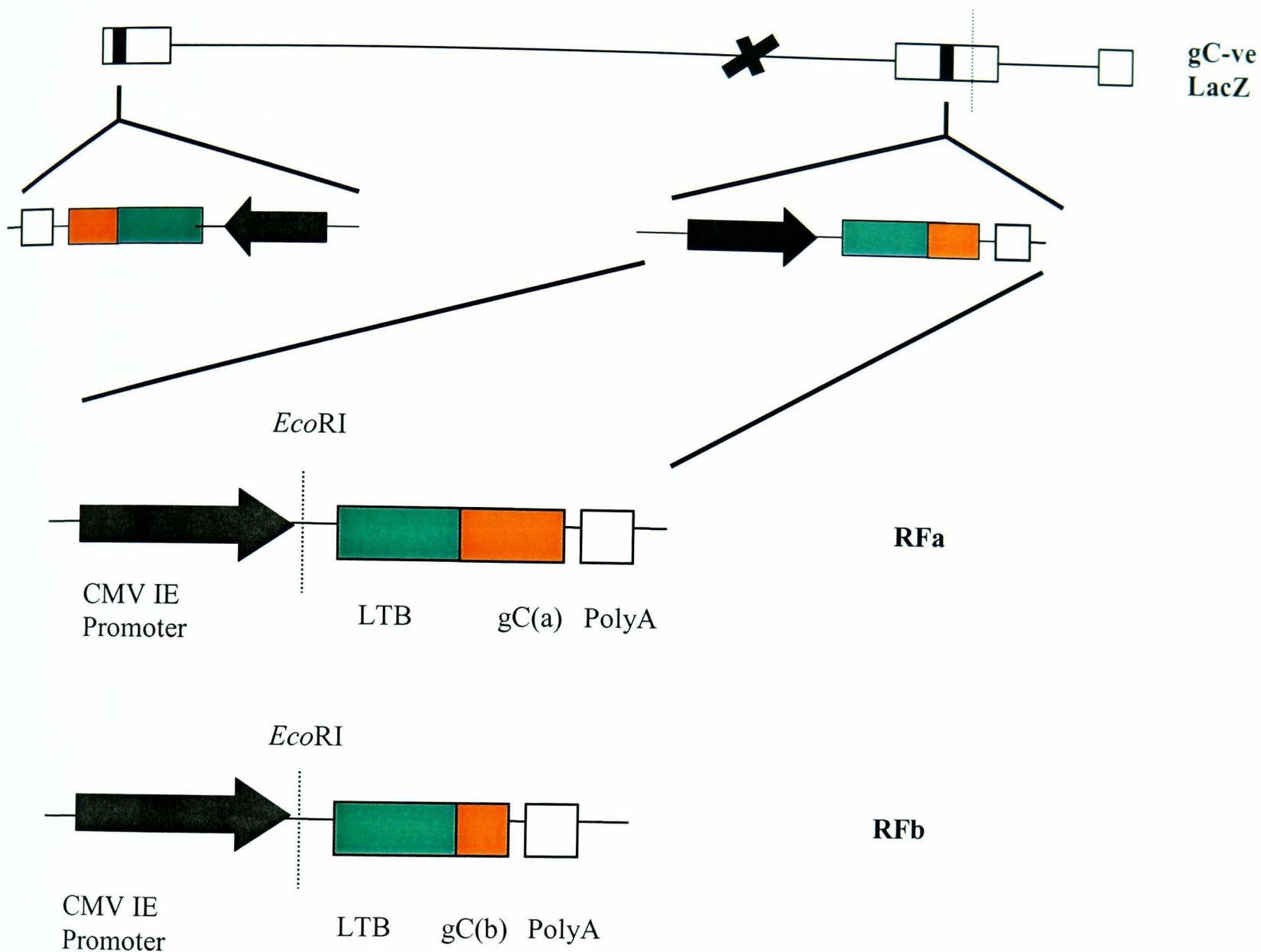
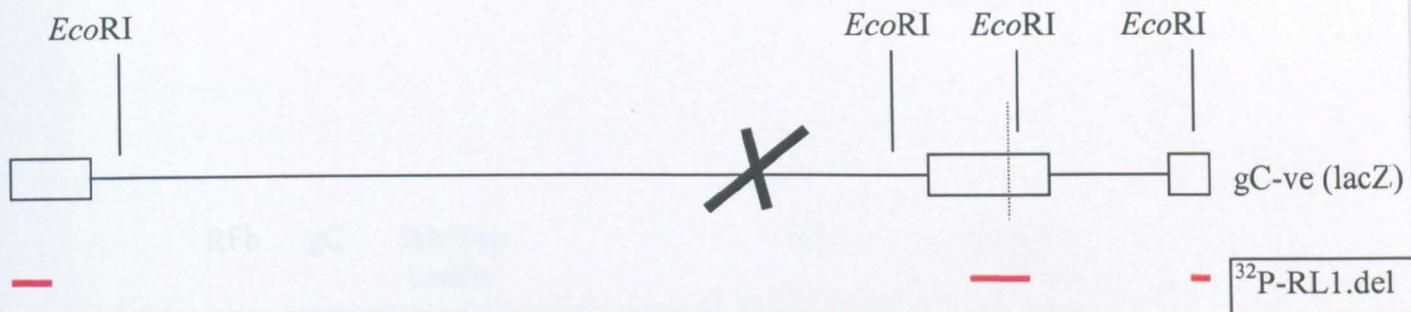


Figure 4.29 Schematic representation of recombinant viruses

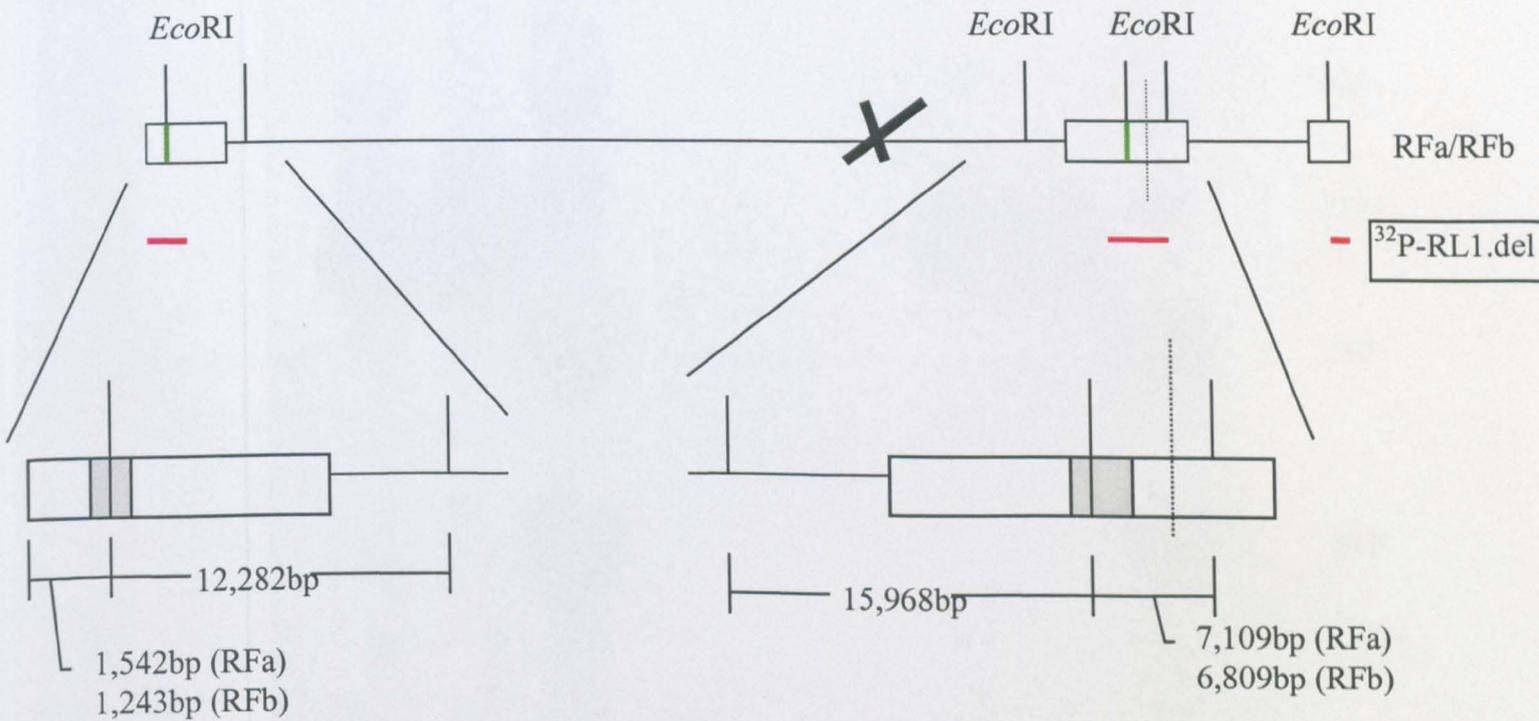
gC-ve (LacZ) was used as the parental strain for the recombinant viruses. Both viruses consist of a LTB-gC gene fusion, containing different sizes of the gC gene, downstream of the CMV IE promoter, and upstream of the HSV-2 IE5 polyadenylation sequence. A novel *EcoRI* site is located between the CMV IE promoter and the LTB-gC gene fusion. They were inserted in the RL1 loci, and thus are present in two copies in the genome. The large X indicates the gC deletion within UL44.



The gC-ve (lacZ) genome was digested with *EcoRI* and probed with ^{32}P -RL1.del during Southern blotting. The *EcoRI* recognition sites are marked above the genome, with the recognition sites of the probe seen as lines beneath the genome.

From Figure 4.27 the following bands are generated and detected by Southern blotting:

- 21,439bp
- 17,553bp
- 16,277bp
- 12,591bp
- 5,566bp



Due to the additional *EcoRI* site, several new bands are generated and recognised by ^{32}P -RL1.del during Southern blotting. Thus, the recombinant viruses give the following bands:

RFa	RFb
17,553bp	17,553bp
15,968bp	15,968bp
12,591bp	12,591bp
12,282bp	12,282bp
7,109bp	6,810bp
5,566bp	5,566bp
1,542bp	1,243bp

Figure 4.30 Schematic diagram of Southern blotting bands for gC-ve, RFa and RfB using a RL1.del probe

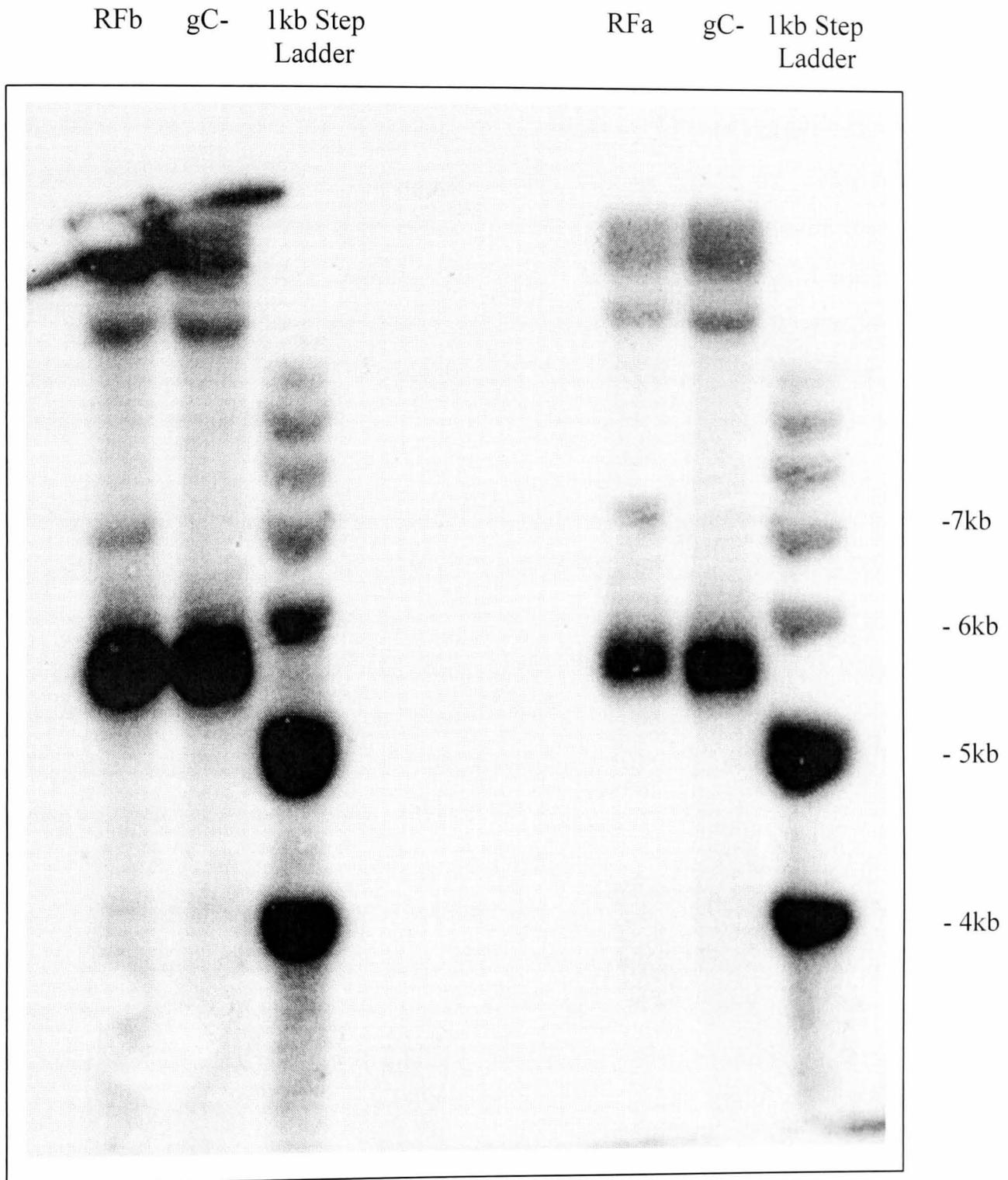


Figure 4.31 Southern blot of purified RFb and partially purified RFa

BHK cells were infected with 17+, RFb or partially purified RFa, and the viral DNA extracted 36h pi. DNA was digested with *Eco*RI and separated on a 0.8% TBE agarose gel. The gel was then Southern blotted using a 32 P-labelled RL1.del probe

4.7 Conclusion

To alter the tropism of HSV-1 from sensory to motor neurons, two recombinant viruses (RFa and RFb) were constructed. To promote binding to GM1 ganglioside, gene fusions of HSV-1 gC and *E.coli* heat-labile enterotoxin were inserted into a HSV-1 gC-ve background. The size of the fusions varied in the degree of truncation of gC. Two different sizes were chosen: One encoding the cytoplasmic and transmembrane domains (amino acids 478-511), which is estimated to be the smallest size of gC which would be incorporated into the membrane (Holland *et al.*, 1988); and a second which encodes the cytoplasmic and transmembrane domains and a region of the extracellular domain (amino acids 377-511). This encodes four cysteine residues that form two disulphide bonds that are suggested to play a role in maintaining the functional conformation of the glycoprotein (Rux *et al.*, 1986).

To generate the recombinant viruses several plasmids were manipulated. The truncated gC genes were amplified by PCR and cloned into pGEM.LTB. This generated fusions of gC with the C-terminal of LTB. The plasmids were sequenced and the gene fusions were seen to be in-frame. These gene fusions were further subcloned into the RL1 locus within plasmid RL1.del, and placed under the control of the CMV IE promoter. The HSV-2 IE5 polyadenylation sequence was then cloned immediately downstream of the gene fusions, generating plasmids pRFa and pRFb.

pRFa and pRFb were then used in a co-transfection with gC-ve (LacZ) DNA to generate mutant viruses RFa and RFb, respectively. Recombinant viruses were detected by *EcoRI* digestion followed by Southern blotting using ³²P-labelled RL1.del. The recombinant viruses have an additional *EcoRI* recognition site located between the CMV IE promoter and the LTB-gC fusion. Using a RL1.del probe, novel bands of 7.1kb and 1.5kb (RFa) or 6.8kb and 1.2kb (RFb) not seen in the parental virus are detected. The purity of the viruses can be seen by Southern blotting as *EcoRI* b (21.4kb) is digested into two bands of 15.9kb and 7.1kb or 6.8kb. Thus if any background contaminating virus was present in the mutant viruses, the 21.4kb band would be present.

A positive recombinant virus was detected from the co-transfection of pRFb and gC-ve (LacZ) DNA. This was plaque-purified three times to ensure purity, before a large-scale stock was grown up on BHK cells.

Similarly a positive recombinant virus was detected for RFa. This however was unable to be purified to homogeneity. Reasons for this will be discussed in chapter 6.

5. CHARACTERIZATION OF RFa & RFb

5.1 Introduction

Following construction of mutant virus RFb and the partial purification of RFa, the characteristics of these viruses were investigated. Viral expression of the LTB-gC fusion was detected by two monoclonal antibodies, 118-8 and LDS47. 118-8 recognises the pentameric, and biologically active form, of LTB and is used in Western blots against unboiled protein samples, while LDS47 recognises the monomeric form of LTB. Immunofluorescence using these antibodies also determined where these proteins were expressed in the cell. The growth characteristics of the viruses were determined on several cell lines to see what effect if any the novel glycoprotein would have on virus binding and replication. The rate of adsorption of virus to various cells, including the motor neuron cell-lines was ascertained. Finally, the rate of viral binding to heparin and several glycolipids was determined by ELISA.

5.2. gC Expression

Glycoprotein C has shown to be the principal glycoprotein involved in binding of HSV-1 to cell surface HS (Fuller and Spear, 1985; Herold *et al.*, 1991; Svennerholm *et al.*, 1991). The regions of gC which bind to HS have been mapped to two distinct regions: one including Arg-143, -145, -147, and Thr-150, and the other containing Gly-247 (Trybala *et al.*, 1994). To promote binding of ganglioside GM1 rather than HS, the mutant viruses were constructed from a gC-negative (17⁺) parental strain.

To confirm virus RFb was indeed gC-ve, BHK cells were infected and harvested 16h pi in SDS lysis buffer and analysed by 12.5% SDS-PAGE and Western blotted with gC antiserum (Figure 5.1). Glycoprotein C was detected from 17⁺ infected extracts, with no gC-expression detected from either RFb or its parental virus, gC-ve (LacZ).

5.3 ICP34.5 Expression

The gene product of RL1, ICP34.5 has been shown to be the major determinant of neurovirulence in HSV-1 (Thompson *et al.*, 1983). Mutants in this region have been shown to be considerably less virulent than wild type virus (Taha *et al.*, 1989; Chou *et al.*, 1990; MacLean *et al.*, 1991). To ensure the non-neurovirulence of RFb the LTB-gC gene fusion was inserted into the RL1 locus. Lack of gene expression from this locus was determined by Western blotting using antisera against ICP34.5 (Figure 5.2). ICP34.5 was detected from 17+ infected extracts, with none detected from RFb or 1716 (an RL1 null mutant). The 70kDa virus induced protein of unknown origin, which is detected by Ab137, is present in all viral samples (Brown *et al.*, 1997).

5.4 LTB-gC Gene Expression in RFb

Recombination of the DNA encoding the LTB-gC fusion molecules with viral DNA may lead to redirected virus infection through recognition of various gangliosides only if the recombinant glycoproteins are appropriately expressed and incorporated into the viral envelope. Thus BHK cells were infected with the recombinant viruses and analysed for the presence of LTB-gC fusions by Western blotting and immunofluorescence.

5.4.1 Western Blotting

BHK cells were infected with RFb and harvested 16h pi. Samples were harvested in two ways. To detect the monomeric form of LTB, cells were harvested in SDS lysis buffer and boiled for 10 min prior to loading on a SDS-PAGE gel. This results in dissociation of any LTB pentamers into its monomeric form. This was then Western blotted using monoclonal antibodies LDS47 or LDS102. To detect the pentameric form samples were harvested in a manner to avoid dissociation of the proteins (section 3.35) and separated by non-denaturing PAGE. This was then detected by Western blotting using monoclonal antibody 118-8.

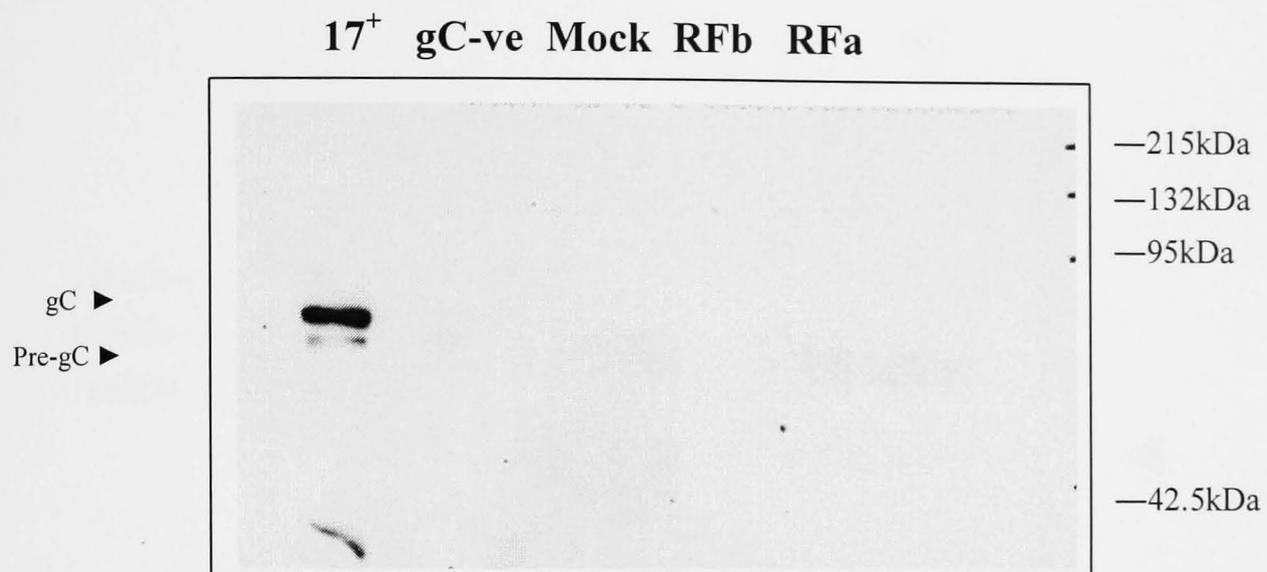


Figure 5.1 Western blot using anti-gC antisera

Protein samples were run on a 12.5% SDS-PAGE and blotted using anti-gC antisera. Two bands (gC and pre-gC) were seen in the wild type lane, but no gC bands were seen in the mock-infected lane, virus RFb or its parental virus gC-ve.

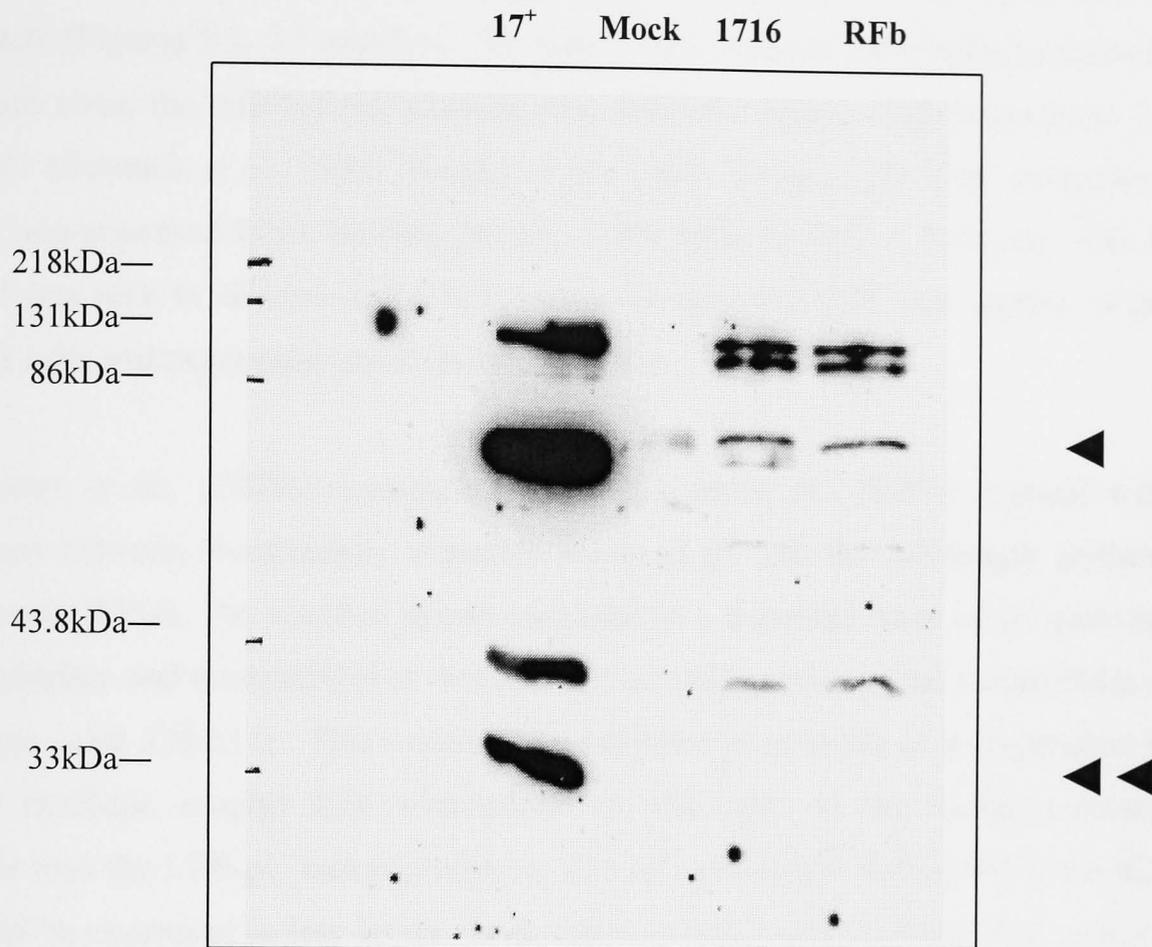


Figure 5.2 Western blot using 137 anti-ICP34.5 antisera

Protein samples were run on a 12.5% SDS-PAGE, blotted and reacted with Ab137, anti-ICP34.5 polyclonal antiserum. ICP34.5 showed up only in the wild type lane (indicated by double arrow head). The 70kDa protein showed up in all viral samples (indicated by arrow head).

Following Western blotting no LTB-gC fusion protein was detected from RFB-infected extracts (Figures 5.1, 5.3 and 5.4). To ensure viral proteins were being expressed by the mutant virus, the infected cell extracts were Western blotted using monoclonal antibody Z1F11 (Schenck *et al.*, 1988; Murphy *et al.*, 1989) (Figure 5.5). This recognises the 65 kDa non-structural DNA binding protein, made early in HSV-1 infection. This 65 kDa band was seen in all virus-infected samples, showing that RFB was capable of infecting BHK cells and expressing genes from its genome.

Laquerre *et al.*, (1998a) recently constructed a series of HSV-1 mutants with gene fusions between N-terminally truncated forms of gC and the full-length erythropoietin hormone (EPO). The smallest fusion consisted of a truncated form of gC containing the cytoplasmic and transmembrane domains and a small portion of the extracellular domain (amino acids 376-511). This mutant virus exhibited poor levels of incorporation into the viral envelope, roughly 20% with respect to wild type. As this fusion is considerably larger than the LTB-gC fusion present in RFB, it was thought that similarly the gC fusion would be expressed in low levels. In an effort to load more viral proteins onto a PAGE gel, samples were harvested as per section 3.33 or 3.35 depending on whether the sample was to be boiled or not, and concentrated using a MICROSEPTM microconcentrator with a 3 kDa molecular weight cut off (MWCO) filter. Following concentration the samples were Western blotted using 118-8 or LDS102. This however did not lead to detection of the fusion proteins (data not shown). To confirm the microconcentrator actually worked, a concentrated RFB sample was loaded onto a SDS-PAGE gel and ran alongside a RFB infected sample harvested in SDS-lysis buffer. This was Coomassie blue stained and dried (Figure 5.6).

As no protein was being detected it was thought that possibly the antibodies being used were not sensitive enough to detect low levels of protein. To determine the levels of protein which the respective antibodies i.e. 118-8, LDS47 and LDS102, could detect, known concentrations of purified LTB were added to SDS-PAGE and non-denaturing PAGE gels and Western blotted (Figures 5.7, 5.8, 5.9). 118-8 was able to detect

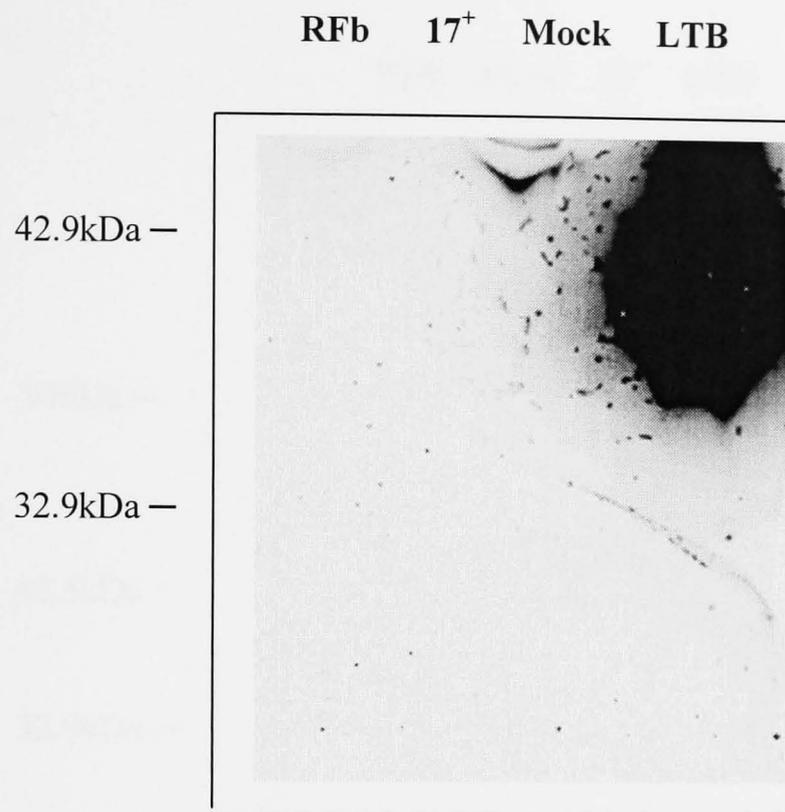


Figure 5.3 Western blot of RFb using 118-8

Protein samples from 60mm plates of BHK cells infected with wild-type and RFb were harvested 24h pi, and loaded, without boiling, onto a 12.5% non-denaturing protein gel. The gel was then blotted against MAb 118-8. As can be seen, no fusion protein was detected in the recombinant virus. 0.5 μ g purified LTB was run alongside as a control.

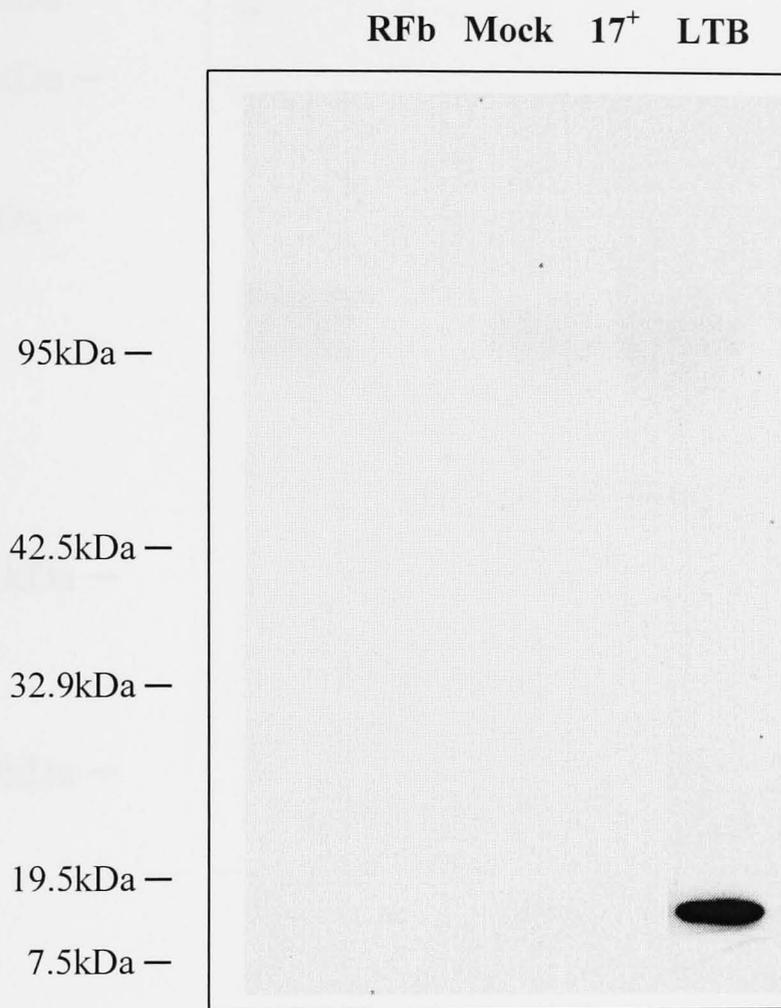


Figure 5.4 Western blot of RFb using 118-8

Protein samples from 60mm plates of BHK cells infected with wild-type and RFb were harvested 24h pi, and loaded, onto a 12.5% denaturing-protein gel. The gel was then blotted against monoclonal antibody LDS102. As can be seen, no fusion protein was detected in the recombinant virus. 0.5 μ g purified LTB was run alongside as a control.

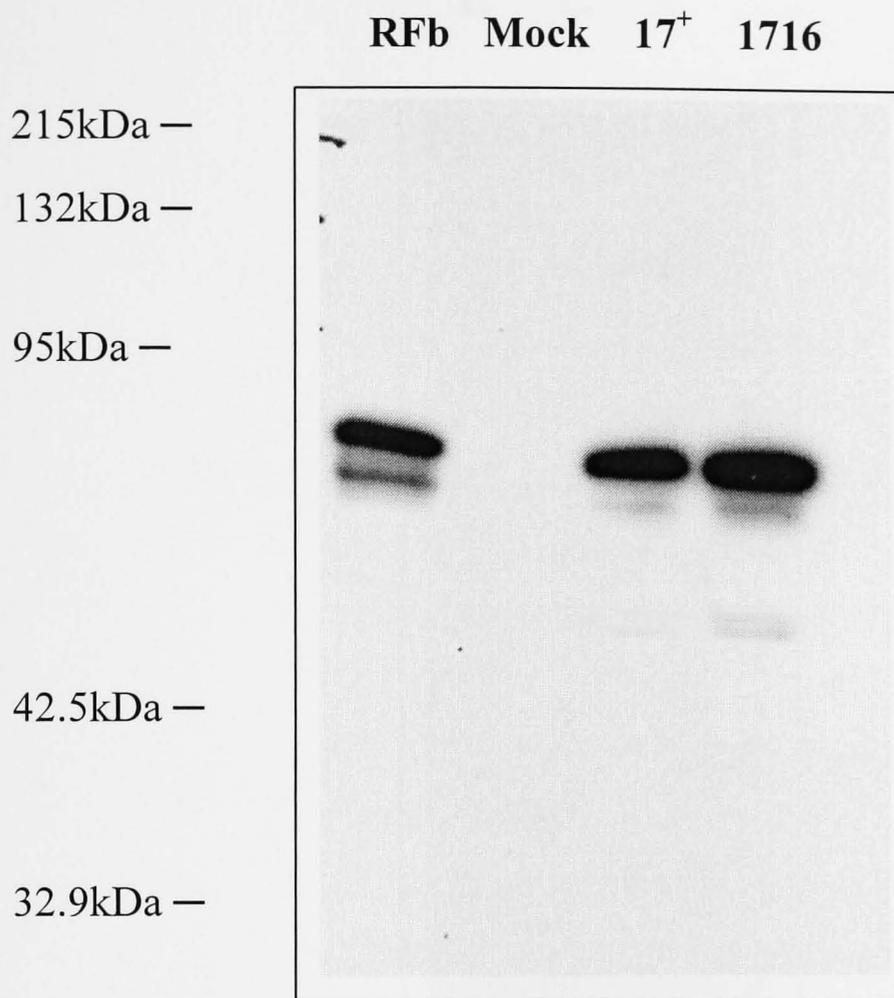


Figure 5.5 Western blot using Z1F11 antibody

Protein samples from wild-type, 1716, RFb and mock-infected cell extracts were run on a 12.5% SDS-PAGE gel and blotted using Z1F11 antibody. The 65kDa nuclear protein is visible in all viral lanes, with none in the mock-infected.

1. 2. 3.

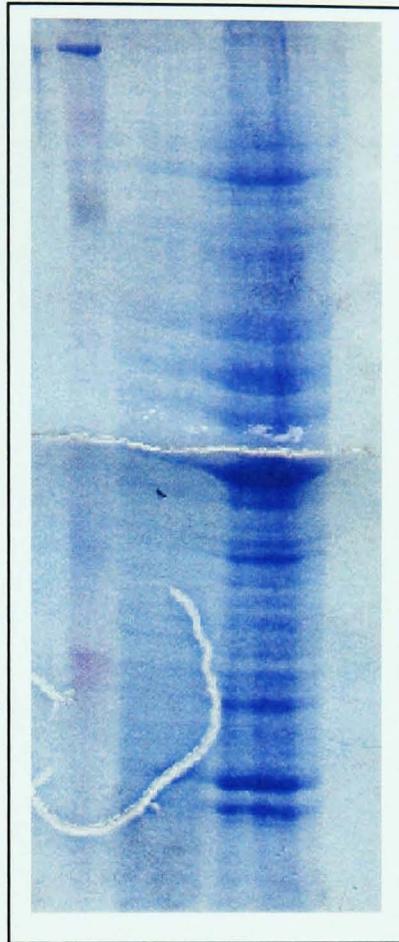


Figure 5.6 Coomassie Blue-Stained SDS-PAGE Gel of RFb viral proteins

Two 60mm plates of BHK cells were infected with RFb. 16h pi the viral proteins were harvested. One plate had its proteins removed in 500 μ l BM. The other had its proteins concentrated in a Microsep 10kDa molecular weight cut-off microconcentrator, which concentrated the sample to 50 μ l. 30ml of both were separated on a 12.5% SDS-PAGE gel and stained with Coomassie Brilliant Blue R250.

Lane 1: Bio-Rad Colour Marker

Lane 2: Unconcentrated RFb sample

Lane 3: Concentrated RFb sample

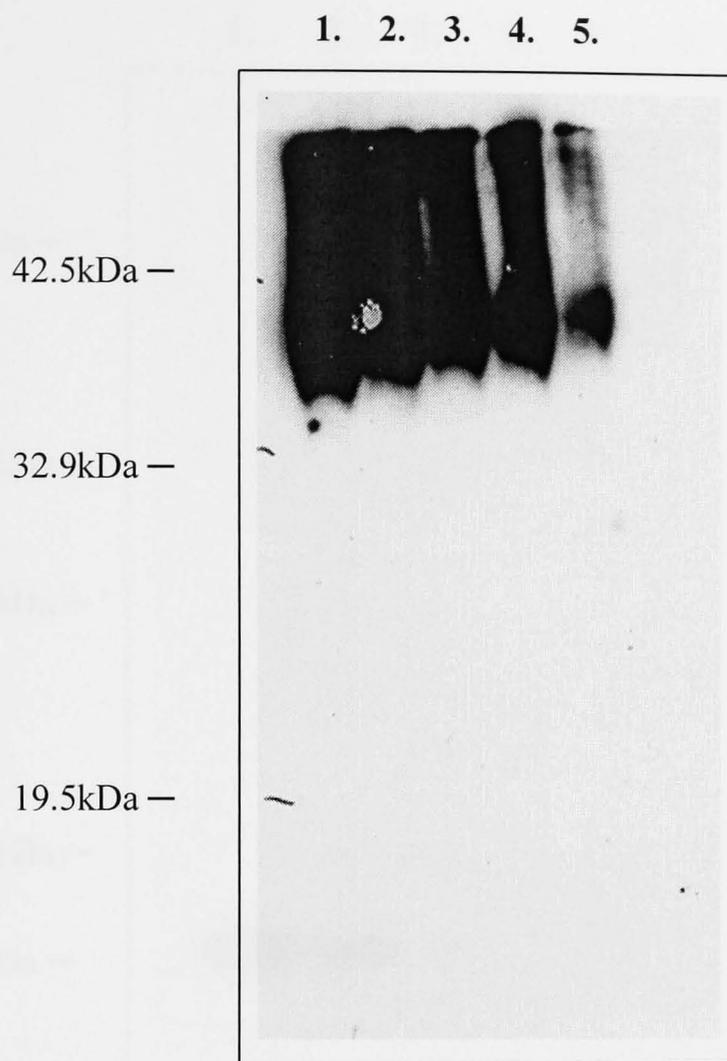


Figure 5.7 Western blot of 118-8 Sensitivity

To ascertain the sensitivity of MAb 118-8 to the pentameric form of LTB, various concentrations of purified LTB protein were run on a 12.5% non-denaturing protein gel, and Western blotted using 118-8. As can be seen from the blot, the MAb can detect protein concentration of 0.01 μ g.

Lane 1: 1 μ g purified LTB Lane 3: 0.1 μ g purified LTB Lane 5: 0.01 μ g purified LTB
 Lane 2: 0.5 μ g purified LTB Lane 4: 0.05 μ g purified LTB

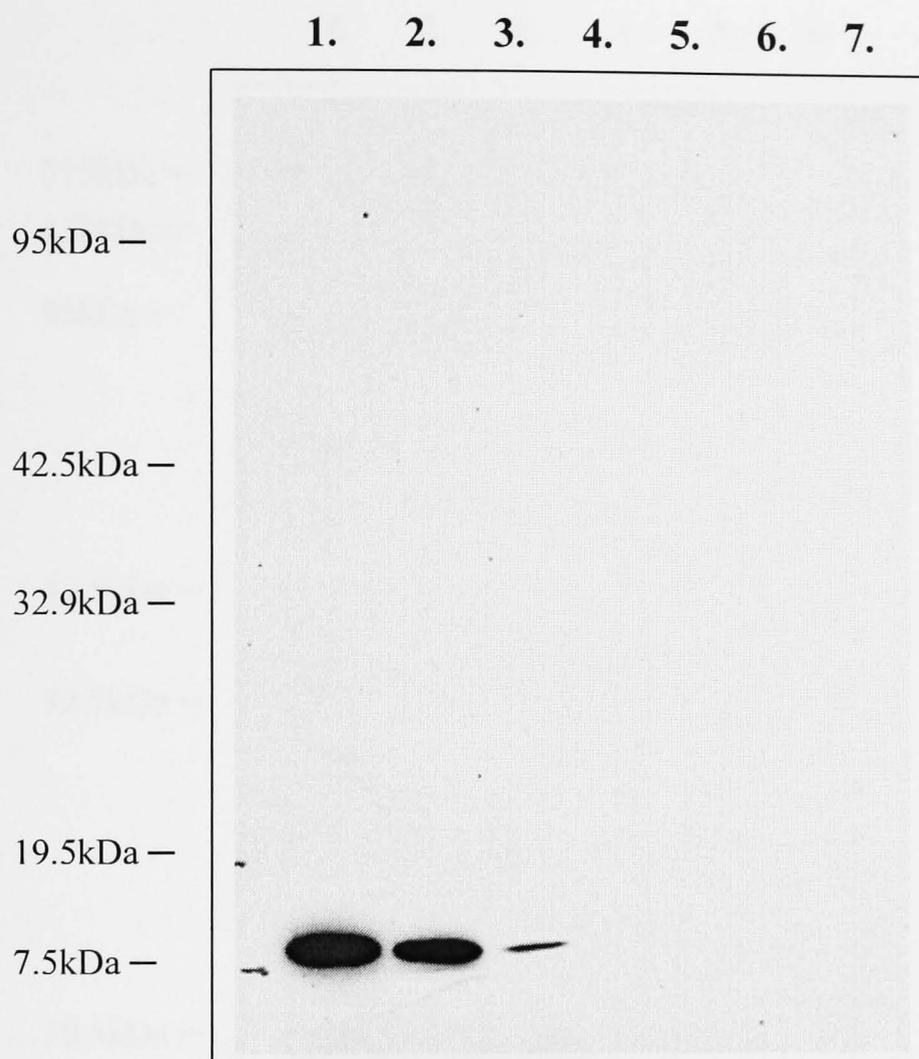


Figure 5.8 LDS47 Sensitivity Assay

To determine the sensitivity of MAb LDS47 various concentrations of LTB were loaded onto a 12.5% SDS-PAGE gel and Western blotted using LDS47. As can be seen the lowest concentration of LTB that the MAb recognises is 1 μ g. Protein concentrations less than this are undetected.

Lane 1: 4 μ g LTB	Lane 3: 1 μ g LTB	Lane 5: 0.1 μ g LTB	Lane 7: 0.01 μ g LTB
Lane 2: 2 μ g LTB	Lane 4: 0.5 μ g LTB	Lane 6: 0.05 μ g LTB	

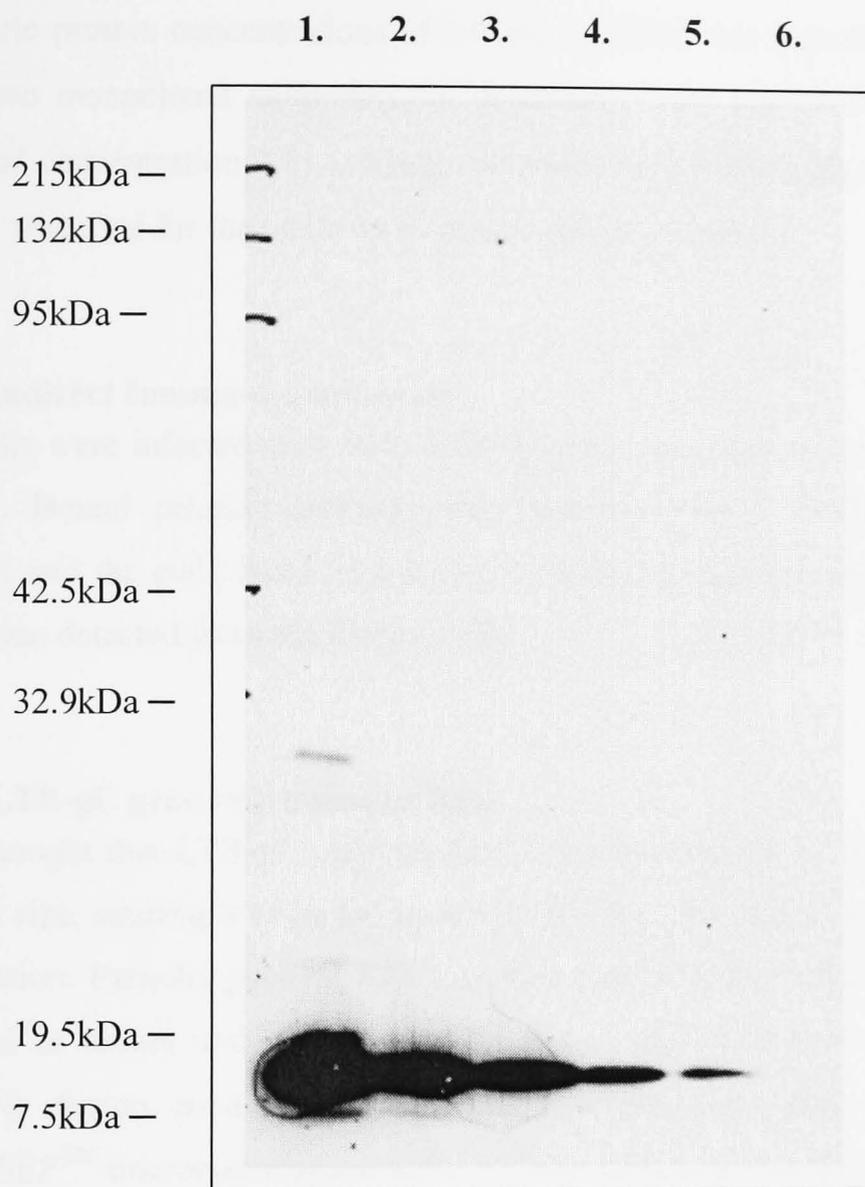


Figure 5.9 LDS102 Sensitivity Assay

To determine the sensitivity of MAb LDS102 various concentrations of LTB were loaded onto a 12.5% SDS-PAGE gel and Western blotted using LDS102. As can be seen the lowest concentration of LTB that the MAb recognises is 0.05 μ g. Protein concentrations less than this are undetected.

Lane 1: 1 μ g LTB Lane 3: 0.25 μ g LTB Lane 5: 0.05 μ g LTB
 Lane 2: 0.5 μ g LTB Lane 4: 0.1 μ g LTB Lane 6: 0.01 μ g LTB

pentameric protein concentrations of 0.01µg. LDS102 was seen to be the more sensitive of the two monoclonal antibodies for detection of the monomer. It was able to bind protein of concentration 0.01-0.05µg, compared to 0.5-0.1µg for LDS47. Consequently, LDS102 was used for the majority of subsequent experiments.

5.4.2 Indirect Immunofluorescence

BHK cells were infected with RFb. LTB epitopes were detected using MAbs 118-8 and LDS102. Bound primary antibody was detected with a TRITC-labelled anti-mouse antibody, and the cells fixed with ice-cold methanol. As seen in Figure 5.10, no fusion protein was detected from transfected cells.

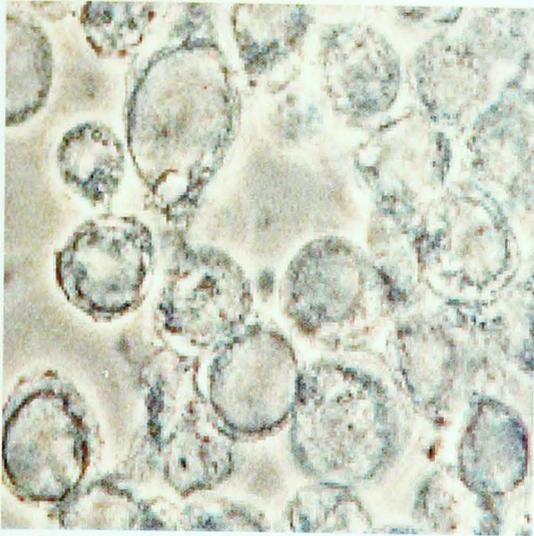
5.5 LTB-gC gene expression in RFa

It was thought that LTB-gC may not have been detected from RFb infected cells due to its small size, causing it to be incorporated into the viral envelope less efficiently than a larger fusion. Partially purified RFa was thus used to infect BHK cells. The cells were harvested as before and Western blotted using 118-8 and LDS102 (Figures 5.11 and 5.12). No fusion protein was detected. Samples were then concentrated using a MICROSEP™ microconcentrator with a 3 kDa molecular weight cut off (MWCO) filter. However, upon Western blotting no protein was detected (data not shown).

5.6 Transfection of pRFa and pRFb

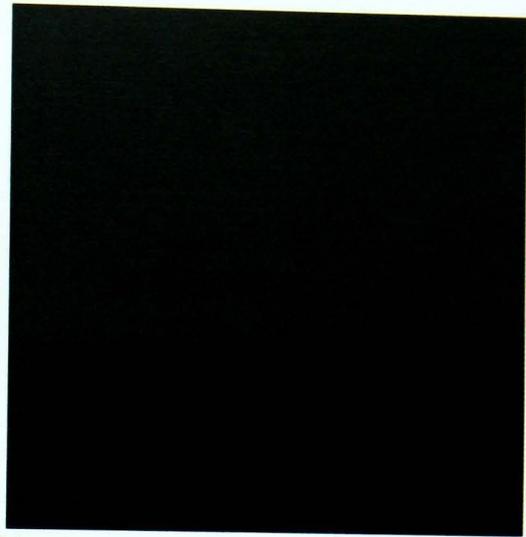
As no fusion protein was detected from either of the mutant viruses, it was decided to analyse BHK cells transfected with plasmids pRFa and pRFb. Fresh, confluent BHK monolayers were transfected with plasmid by either CaPO₄ transfection (section 3.23) or by using a commercially-available kit (section 3.24). Plasmid gene expression was ascertained by indirect immunofluorescence (section 3.36) or by harvesting cell monolayers and Western blotting.

Phase

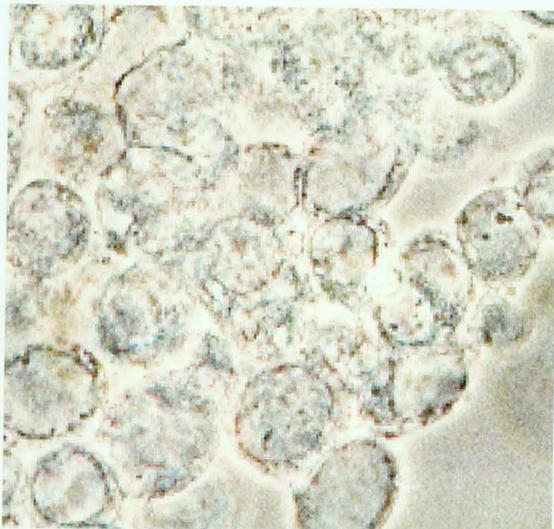


(a)

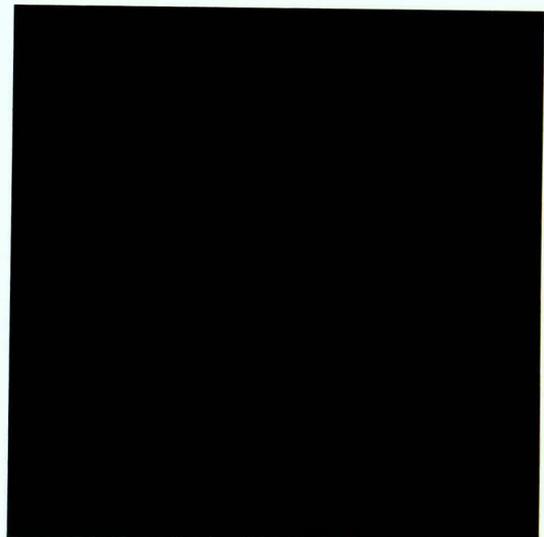
Immunofluorescence



(b)



(c)



(d)

Figure 5.10 RFb-infected BHKs stained with 118-8 and LDS102

BHK cells were mounted on coverslips and infected with RFb. 24h pi cells were incubated using mouse MAb 118-8 (a) & (b) or MAb LSD102 (c) & (d), and then detected using a TRITC-conjugated anti-mouse secondary antibody. Cells were photographed under phase and fluorescent light.

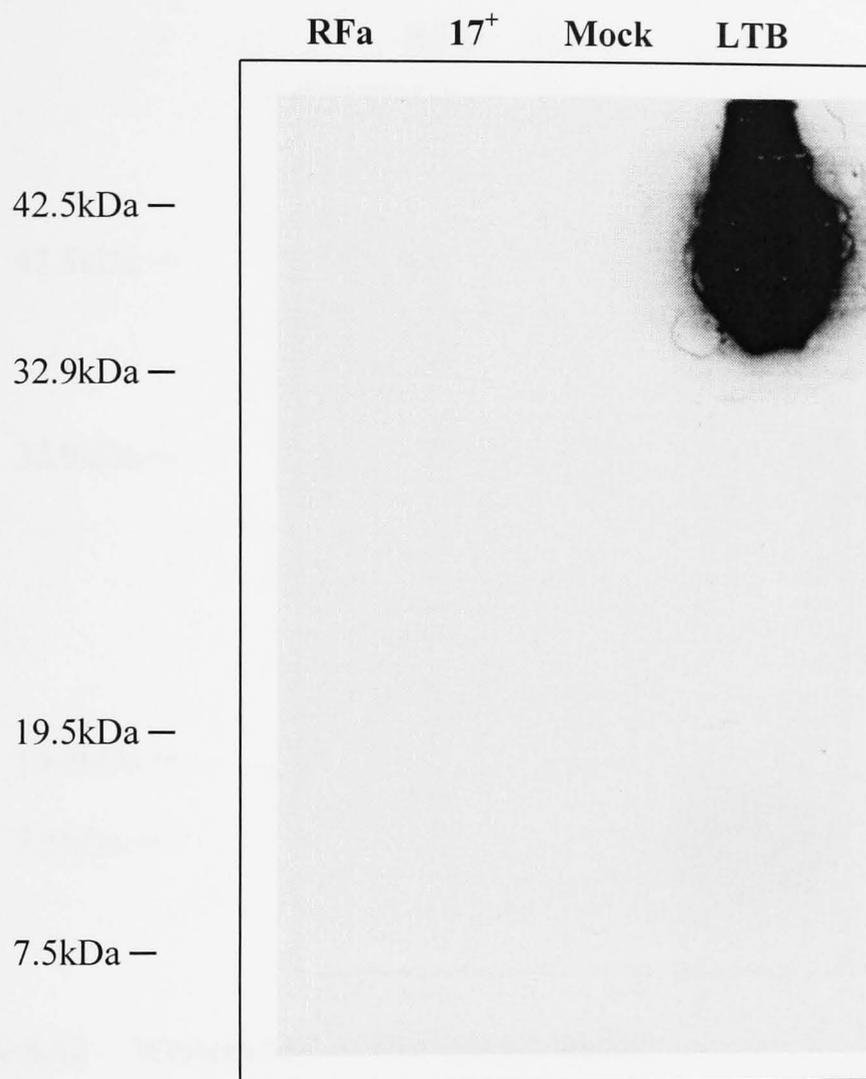


Figure 5.11 Western blot of partially purified RFa against 118-8

A partially purified RFa was used to infect a 60mm plate of BHK cells. The proteins were harvested 24h pi and loaded on a 12.5% non-denaturing protein gel, and Western blotted using MAb 118-8. No fusion protein was detected in the viral sample. 0.5mg of purified LTB was run alongside as control.

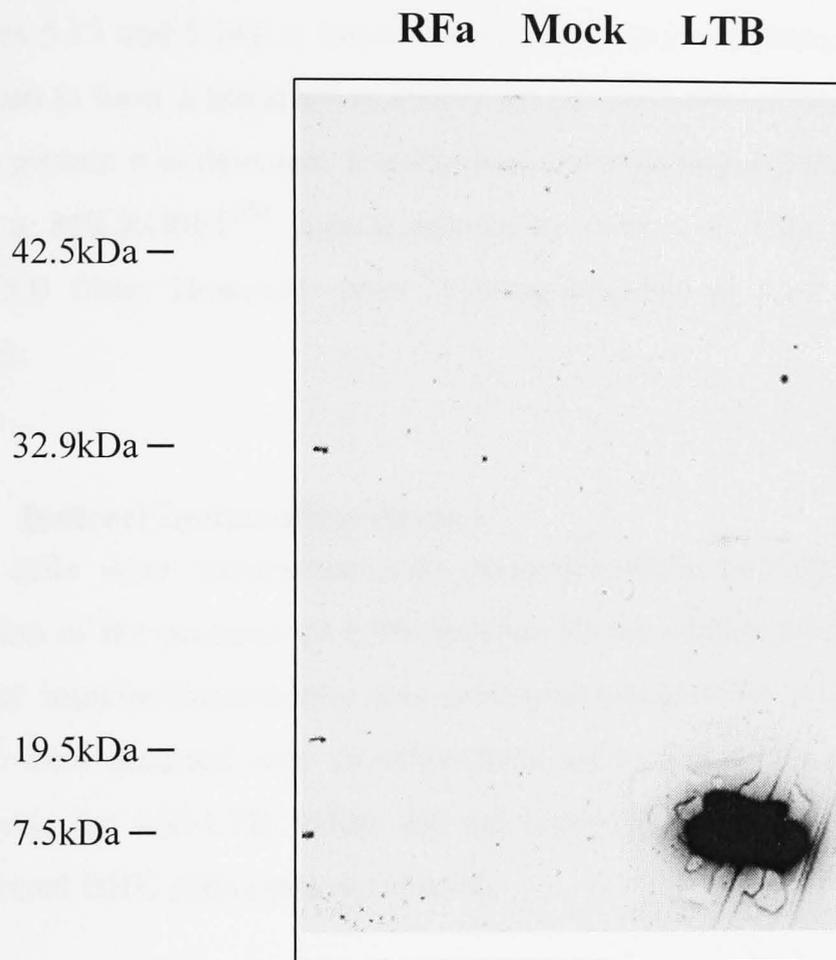


Figure 5.12 Western blot of RFa using LDS102

A partially pure RFa was used to infect a 60mm plate of BHK cells. 24h pi the cells were harvested and ran on a 12.5% SDS-PAGE gel, and Western blotted using MAb LDS102. No fusion proteins were detected in the viral sample. 2 μ g of purified LTB was used as a control (lane 3).

5.6.1 Western Blot Analysis

Confluent BHK cells were transfected with 2 µg, 5 µg or 10µg of purified plasmid. 48h post-transfection cells were harvested and Western blotted using 118-8 and LDS102 (Figures 5.13 and 5.14). A LacZ-containing plasmid was transfected as a control and this was seen to have a transfection efficiency of ~40%. However, from Western blotting, no fusion protein was detected. Transfection was repeated and the cell proteins concentrated using a MICROSEP™ microconcentrator with a 3 kDa molecular weight cut off (MWCO) filter. However, upon Western blotting no protein was detected (data not shown).

5.6.2 Indirect Immunofluorescence

BHK cells were transfected with plasmids pRFa and pRFb. 24h post-transfection, detection of the presence of LTB epitopes on the surface of unfixed transfected cells by indirect immunofluorescence was attempted using MAb 118-8 and LDS102. Bound MAbs were detected with an anti-mouse secondary antibody conjugated with TRITC. However, the anti-LTB MAbs did not recognise any LTB epitopes at the surface of transfected BHK cells (data not shown).

5.7 *In vitro* transcription of LTB.gCb gene fusion

As no fusion protein was being detected by Western blotting or Immunofluorescence, it was possible that this was due to a problem in transcription of the gene fusion. To investigate, the LTB.gCb gene fusion was *in vitro* transcribed using the Ambion MEGAscript T7 Transcription Kit (section 3.41). The RNA transcripts were separated on a 6% Acrylamide/7M Urea gel and stained with ethidium bromide (Figure 5.15). As can be seen the gene fusion gave a RNA transcript of the correct expected size (504bp).

5.8 *In vitro* Replication of RFb

The fact that we were unable to detect LTB-gC fusion proteins from virally-infected cells by Western blot or indirect immunofluorescence was not necessarily indicative of a lack

of fusion gene expression from the viral backbone. This may have been merely a consequence of slight alterations in the tertiary structure of the LTB protein, caused by fusion to gC, thus masking epitope recognition sites from MAbs against LTB.

To determine if incorporation of the LTB-gC fusion protein into the viral envelope could alter the growth characteristics of the mutant virus, we analysed the growth of several different HSV-1 viruses in different cells.

In BHK cells, all viruses grew like wild type (Figure 5.16; Table 1). Using 3T6 cells, a cell line that is non-permissive for RL1-deleted viruses, RFb grew similarly to 1716, a RL1-null mutant, demonstrating that insertion of the gene fusion into the virus had indeed knocked out RL1 gene function *in vitro* (Figure 5.17). In experiments with the motor neuron-like cell lines, NSC-19 and NSC-34, all viruses tested failed to grow as successfully as they had on BHK or 3T6 cells (Figures 5.18 & 5.19). Here viruses had a very small growth burst, typically only one log higher than input. Using NSC-34 cells, RFb and its parental virus gC-ve grew less well than wild type or 1716.

5.9 *In vivo* Adsorption of RFb

The ability of RFb to adsorb to cells *in vivo* was compared to that of wild type and gC-negative virus (section 3.38). During this experiment virus was allowed to adsorb to cells at 37⁰C, rather than 4⁰C, as the motor neuron-like cell lines were sensitive to prolonged incubation at 4⁰C.

On BHK cells all viruses showed similar patterns of adsorption (Figure 5.20; Table 2). gC-negative and RFb both showed a slight lag in the initial stages of binding with respect to wild type, however all virus was adsorbed after 60 min.

All viruses showed a decreased ability to bind to NSC-19 and NSC-34 cells with only 10% of added virus binding (Figures 5.21, 5.22).

1. 2. 3. 4. 5. 6. 7. 8.

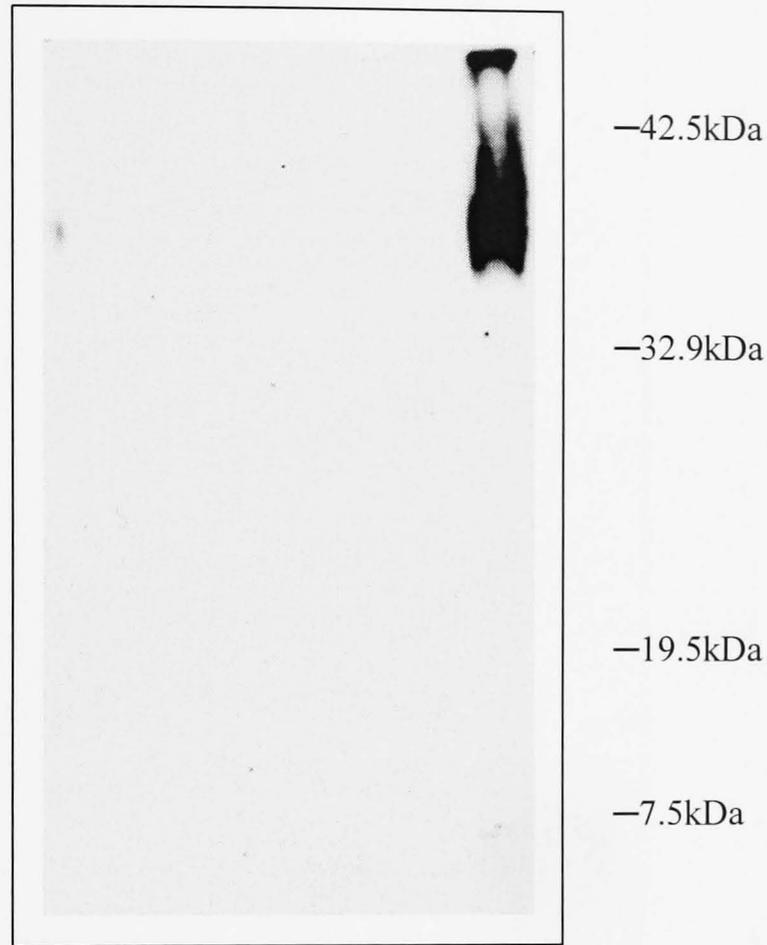


Figure 5.13 Western blot of transfected cell extracts using 118-8

BHK cells were transfected with both pRFa and pRFb at a variety of concentrations. The cell extracts were then ran on a 12.5% SDS-PAGE gel and Western blotted using MAb 118-8, which recognises the pentameric form of LTB. LTB-gC fusion proteins were not detected in the transfected samples, with only control LTB giving a band (lane8).

Lane 1: pRFa (2 μ g)

Lane 4: pRFb (2 μ g)

Lane 7: BHK (untransfected)

Lane 2: pRFa (5 μ g)

Lane 5: pRFb (5 μ g)

Lane 8: LTB control (2 μ g)

Lane 3: pRFa (10 μ g)

Lane 6: pRFb (10 μ g)

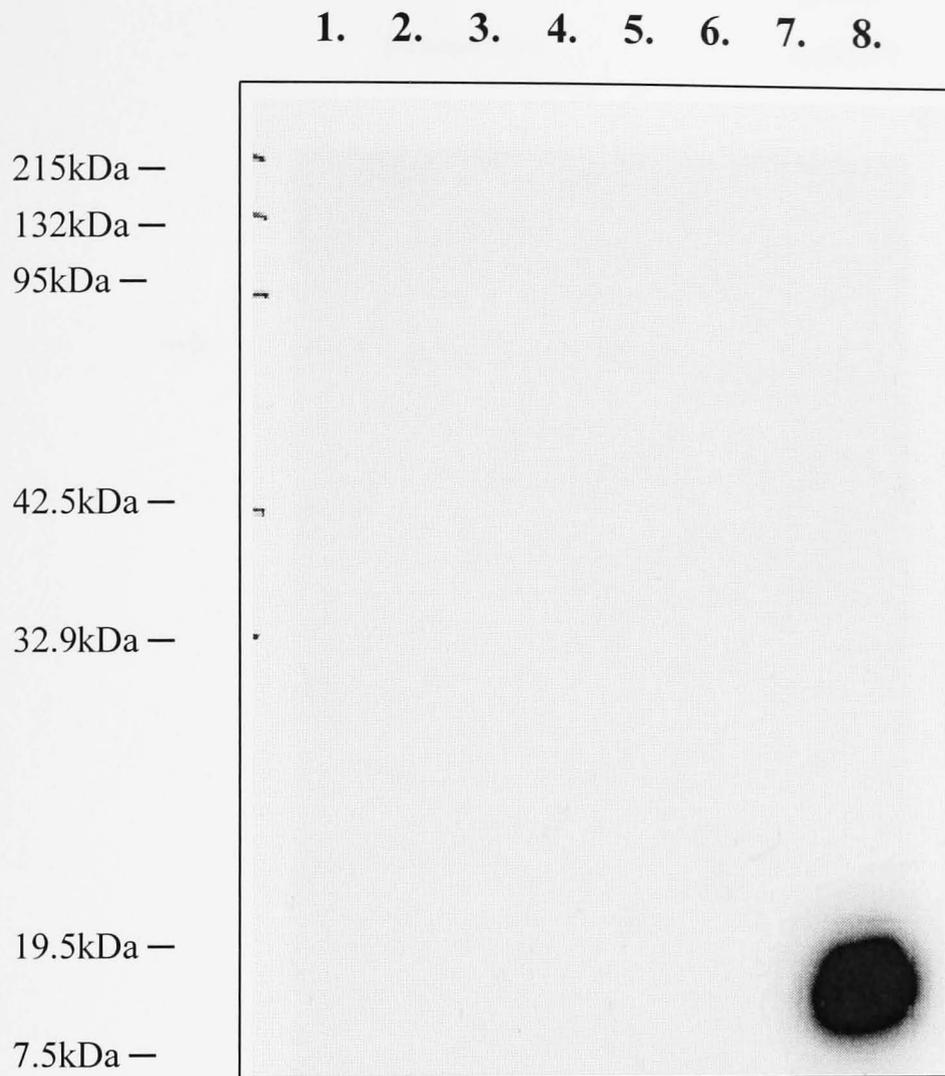


Figure 5.14 Western blot of transfected cell extracts using LDS102

BHK cells were transfected with both pRFa and pRFb at a variety of concentrations. The cell extracts were then ran on a 12.5% SDS-PAGE gel and Western blotted using MAb LDS102, which recognises the monomeric form of LTB. LTB-gC fusion proteins weren't detected in the transfected samples, with only control LTB giving a band (lane8).

Lane 1: pRFa (2 μ g)

Lane 4: pRFb (2 μ g)

Lane 7: BHK (untransfected)

Lane 2: pRFa (5 μ g)

Lane 5: pRFb (5 μ g)

Lane 8: LTB control (2 μ g)

Lane 3: pRFa (10 μ g)

Lane 6: pRFb (10 μ g)

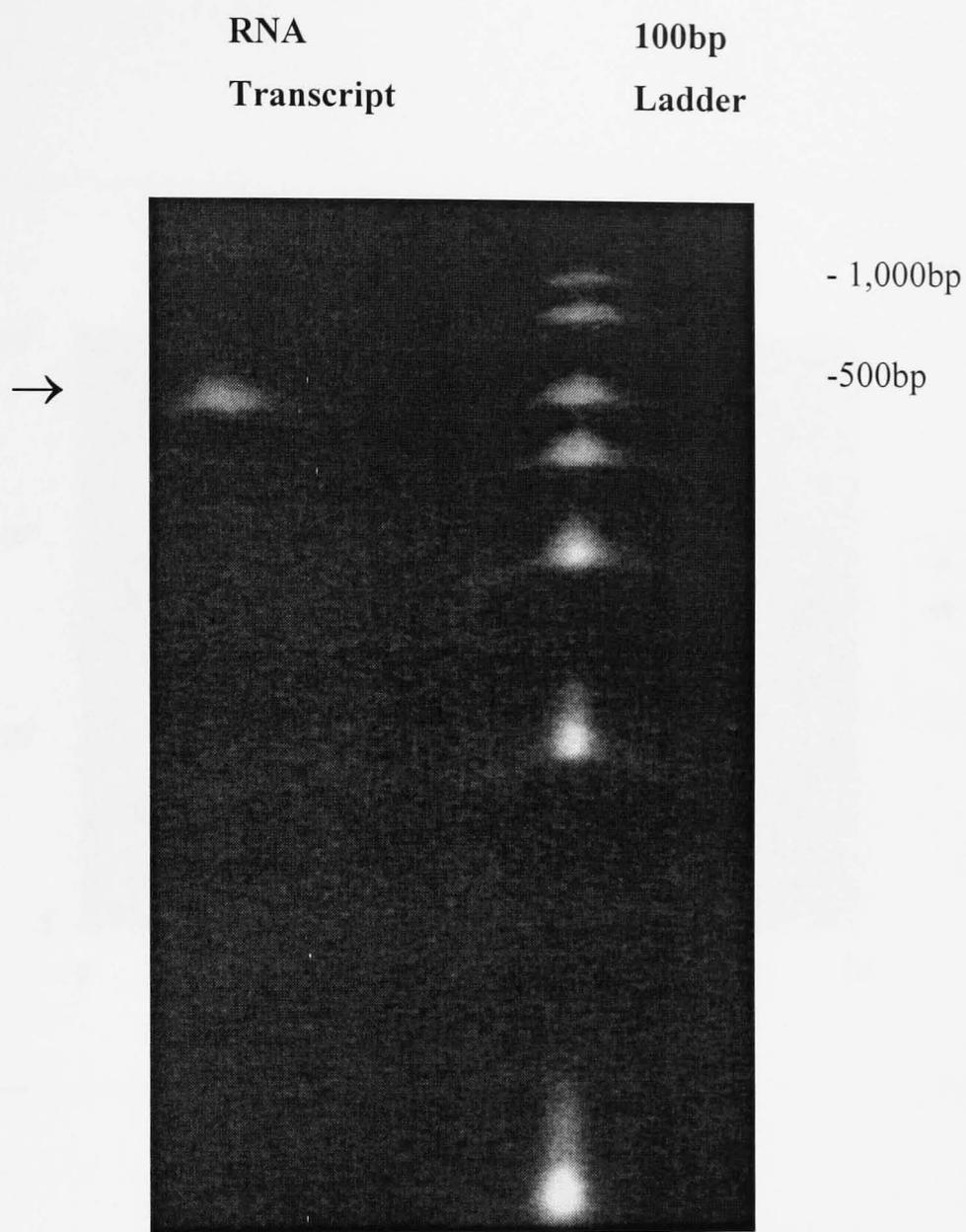


Figure 5.15 *In vitro* transcription of pGEM.LTB.gC(b)

Using the Ambion (Europe) Ltd. MEGAscript T7 Transcription Kit, RNA was synthesised from the DNA template pGEM.LTB.gC(b), separated on a 6% Acrylamide/7M Urea gel and stained with ethidium bromide. The 504bp transcript can be seen indicated by arrow.

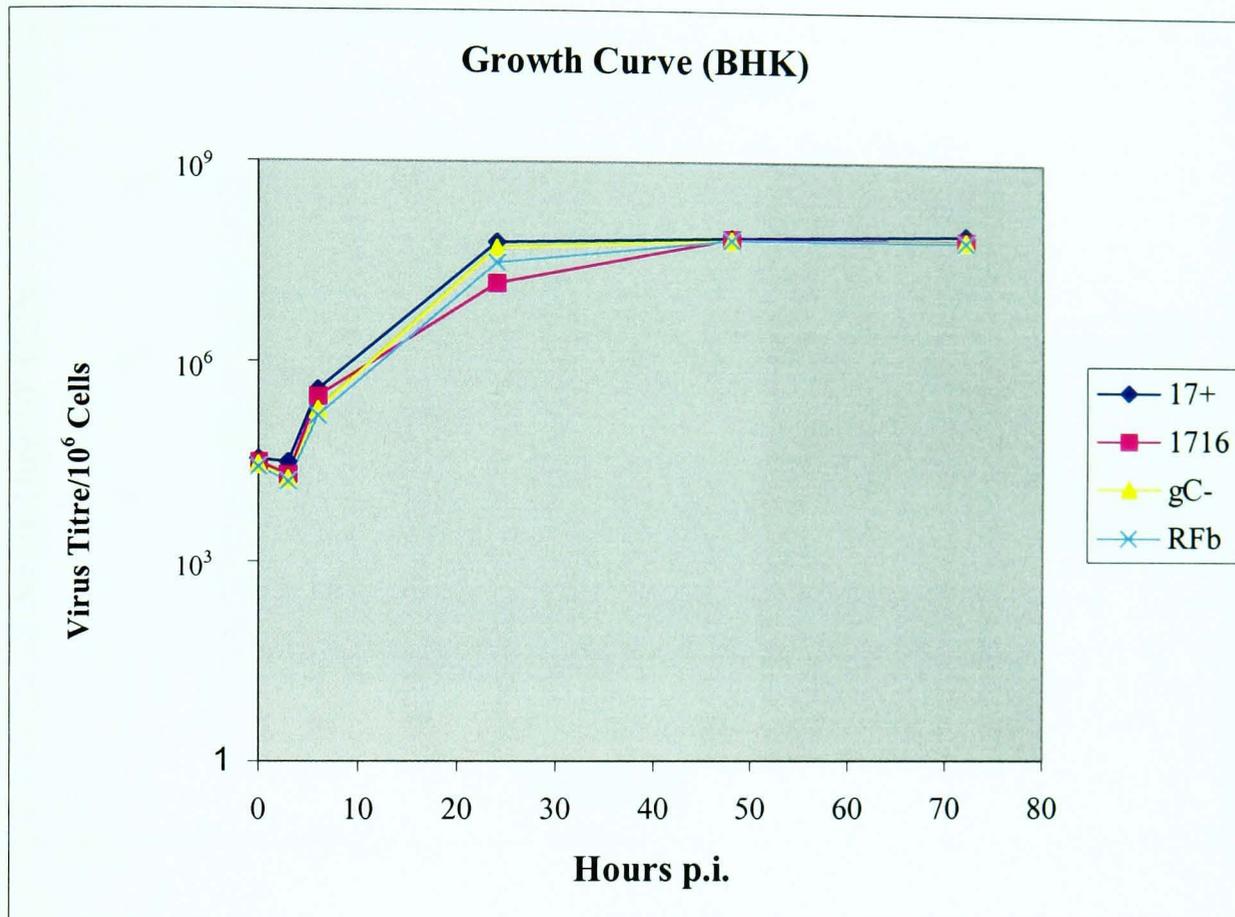


Figure 5.16 *In vitro* growth of 17⁺, 1716, gC-ve and RFb in BHK cells

BHK cells were infected at a moi of 0.01 pfu/cell. Infected cells were harvested at 0, 3, 6, 24, 48 and 72 h pi. Infected cells were harvested, sonicated and virus titrated on BHK cell monolayers.

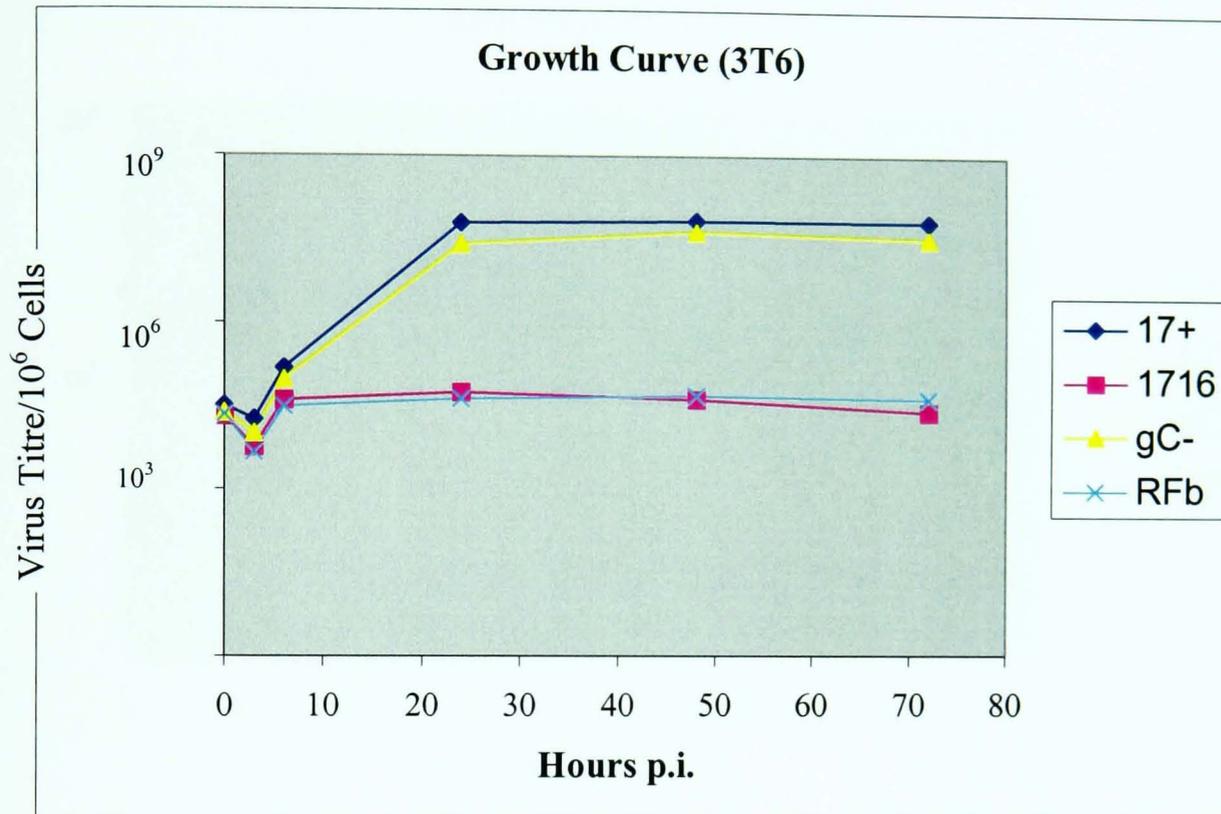


Figure 5.17 *In vitro* growth of 17⁺, 1716, gC-ve and RFb in 3T6 cells

3T6 cells were infected at a moi of 0.01 pfu/cell. Infected cells were harvested at 0, 3, 6, 24, 48 and 72 h pi. Infected cells were harvested, sonicated and virus titrated on BHK cell monolayers.

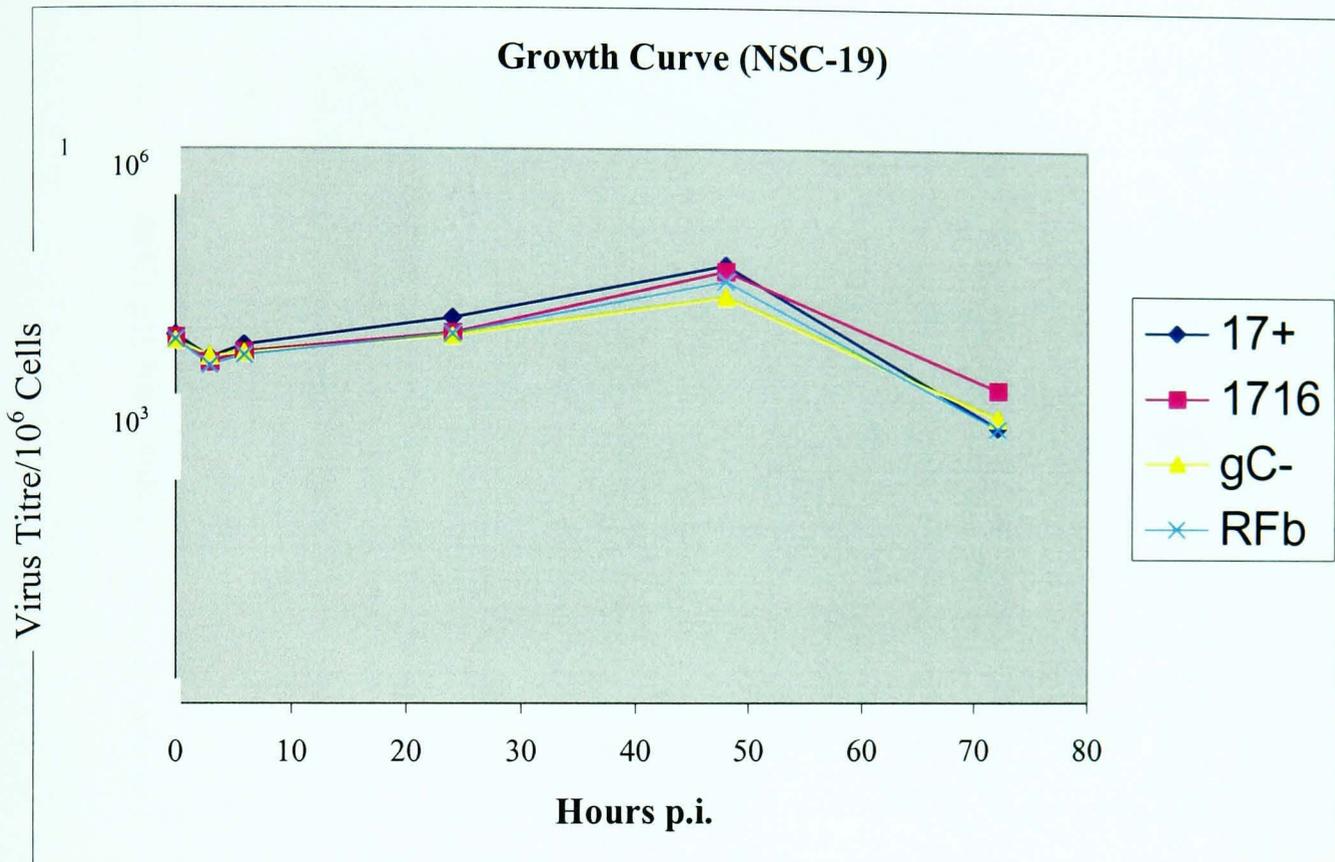


Figure 5.18 *In vitro* growth of 17⁺, 1716, gC-ve and RFb in NSC-19 cells

NSC-19 cells were infected at a moi of 0.01 pfu/cell. Infected cells were harvested at 0, 3, 6, 24, 48 and 72 h pi. Infected cells were harvested, sonicated and virus titrated on BHK cell monolayers.

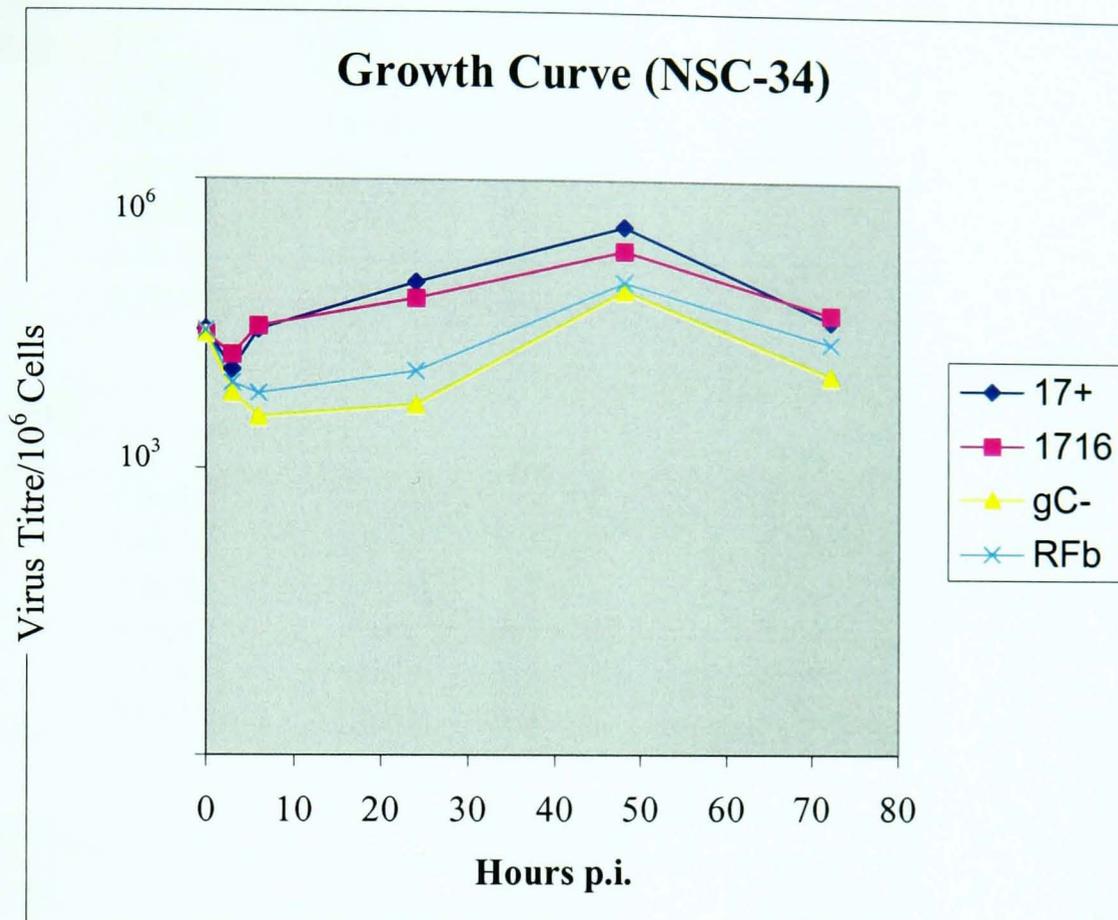


Figure 5.19 *In vitro* growth of 17⁺, 1716, gC-ve and RFb in NSC-34 cells

NSC-34 cells were infected at a moi of 0.01 pfu/cell. Infected cells were harvested at 0, 3, 6, 24, 48 and 72 h pi. Infected cells were harvested, sonicated and virus titrated on BHK cell monolayers.

3T6 cells				
Time(h)	17+	1716	gC-ve	RFb
0	3.2×10^4	2.1×10^4	2.4×10^4	2.2×10^4
3	1.8×10^4	5.9×10^4	1.0×10^4	4.5×10^3
6	1.5×10^5	4.0×10^4	9.5×10^4	3.0×10^4
24	6.2×10^7	5.5×10^4	2.5×10^7	4.2×10^4
48	6.8×10^7	4.1×10^4	4.2×10^7	4.8×10^4
72	6.5×10^7	2.5×10^4	3.2×10^7	4.2×10^4

BHK cells				
Time (h)	17+	1716	gC-ve	RFb
0	3.5×10^4	3.0×10^4	2.9×10^4	2.7×10^4
3	3.0×10^4	2.0×10^4	1.7×10^4	1.5×10^4
6	3.8×10^5	3.0×10^5	1.9×10^5	1.5×10^5
24	6.3×10^7	1.5×10^7	5.0×10^7	3.1×10^7
48	7.1×10^7	7.0×10^7	6.7×10^7	6.7×10^7
72	8.0×10^7	6.7×10^7	6.7×10^7	6.5×10^7

NSC-19 cells				
Time (h)	17+	1716	gC-ve	RFb
0	9.8×10^3	9.4×10^3	8.5×10^3	8.8×10^3
3	5.5×10^3	5.0×10^3	5.8×10^3	4.5×10^3
6	7.5×10^3	6.5×10^3	6.3×10^3	5.9×10^3
24	1.5×10^4	1.0×10^4	9.5×10^3	1.0×10^4
48	5.5×10^4	4.8×10^4	2.5×10^4	3.8×10^4
72	1.0×10^3	2.5×10^4	1.3×10^4	9.5×10^4

NSC-34 cells				
Time (h)	17+	1716	gC-ve	RFb
0	2.7×10^4	2.4×10^4	2.4×10^4	2.6×10^4
3	1.0×10^4	1.5×10^4	5.8×10^3	7.5×10^3
6	2.7×10^4	3.0×10^4	3.2×10^3	5.9×10^3
24	8.5×10^4	5.9×10^4	4.5×10^3	1.0×10^4
48	3.3×10^5	1.9×10^5	6.9×10^4	8.5×10^4
72	3.5×10^4	4.1×10^4	9.0×10^3	2.0×10^4

Table 1. 3T6, BHK, NSC-19 and NSC-34 titres for single cycle growth kinetics

Titres given as p.f.u. per 10^6 cells

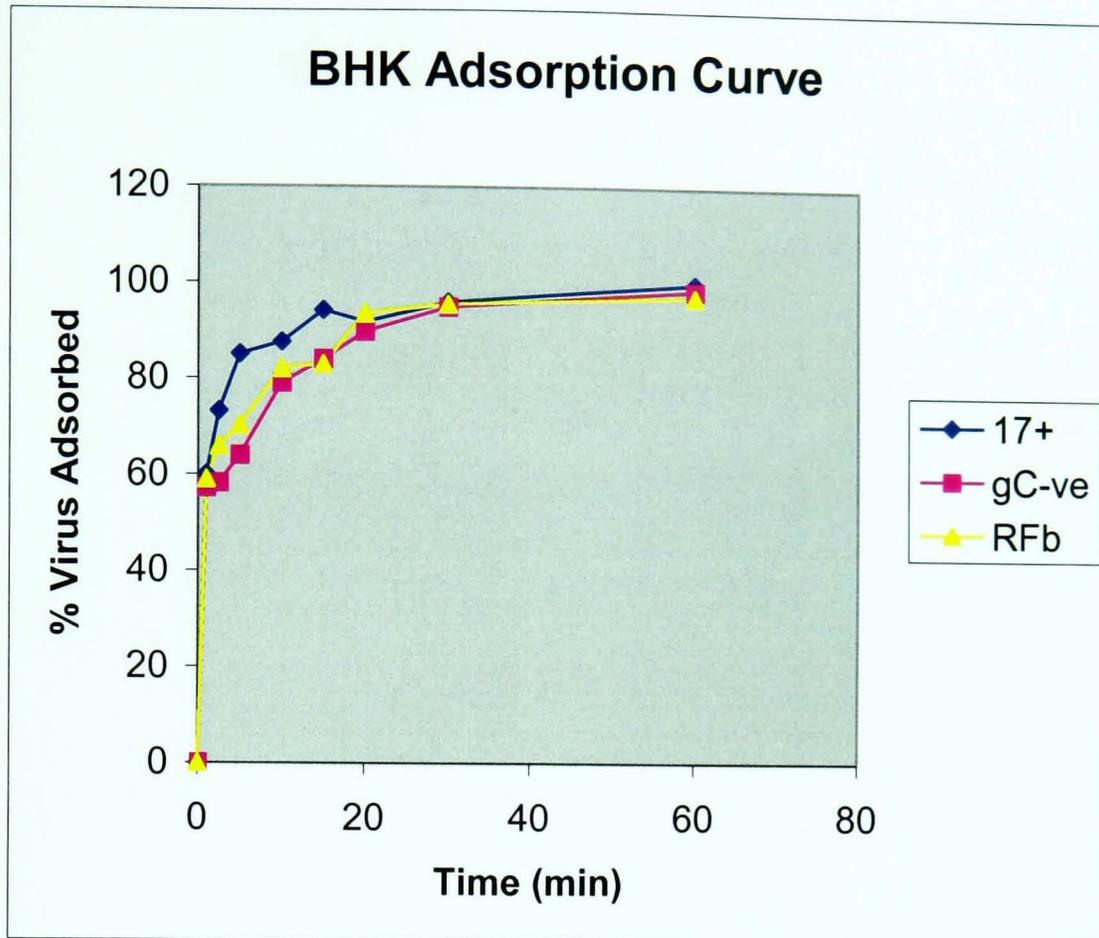


Figure 5.20 *In vivo* adsorption of 17⁺, gC-ve and RFb to BHK cells

BHK cells were infected at a moi of 500 pfu/plate. Virus was allowed to adsorb to cells at 37⁰C. At 0, 1, 2.5, 5, 10, 15, 20, 30 and 60 min unbound virus was washed off, and cells overlaid with growth medium. Following 24h incubation at 37⁰C media was removed and cells stained for LacZ expression. Cells were counted, whereby each blue cell represented one bound virus.

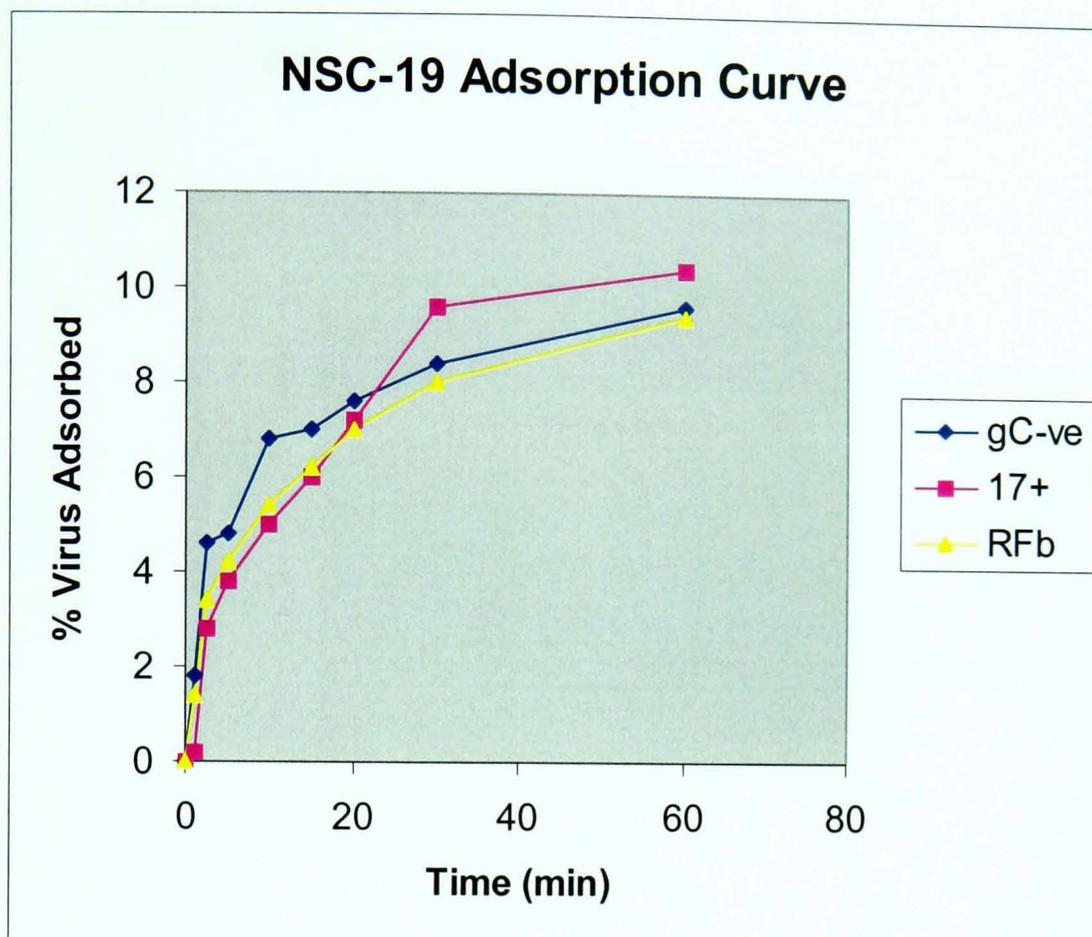


Figure 5.21 *In vivo* adsorption of 17⁺, gC-ve and RFb to NSC-19 cells

NSC-19 cells were infected at a moi of 500 pfu/plate. Virus was allowed to adsorb to cells at 37⁰C. At 0, 1, 2.5, 5, 10, 15, 20, 30 and 60 min unbound virus was washed off, and cells overlaid with growth medium. Following 24h incubation at 37⁰C media was removed and cells stained for LacZ expression. Cells were counted, whereby each blue cell represented one bound virus.

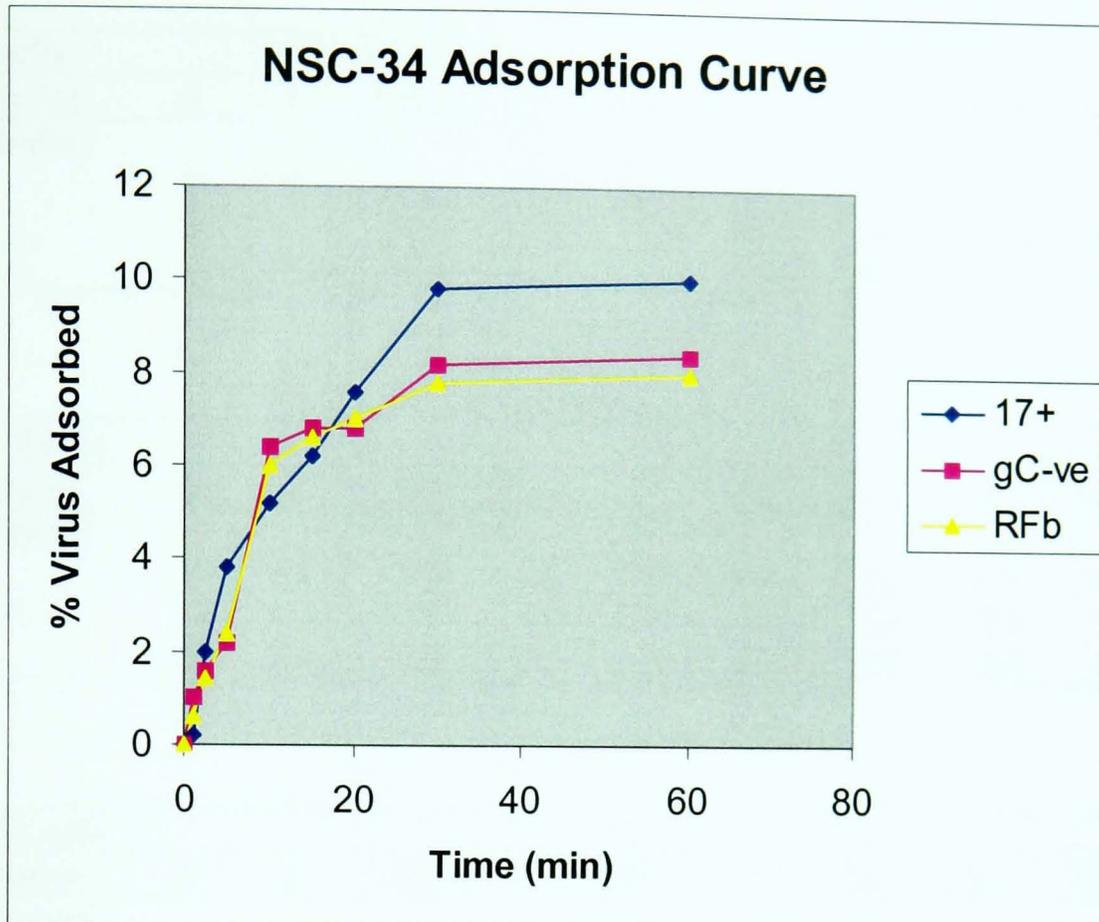


Figure 5.22 *In vivo* adsorption of 17⁺, gC-ve and RFb to NSC-34 cells

NSC-34 cells were infected at a moi of 500 pfu/plate. Virus was allowed to adsorb to cells at 37⁰C. At 0, 1, 2.5, 5, 10, 15, 20, 30 and 60 min unbound virus was washed off, and cells overlaid with growth medium. Following 24h incubation at 37⁰C media was removed and cells stained for LacZ expression. Cells were counted, whereby each blue cell represented one bound virus.

BHK cells									
Time (min)	0	1	2.5	5	10	15	20	30	60
% Adsorbed									
17 ⁺	0	60.0	73.2	85.0	87.6	94.2	91.8	96.0	100
gC-ve	0	57.2	58.2	64.0	79.0	84.0	89.6	95.0	98.6
RFb	0	59.0	65.8	70.2	82.0	83.0	93.6	95.6	97.2

NSC-19 cells									
Time (min)	0	1	2.5	5	10	15	20	30	60
% Adsorbed									
17 ⁺	0	0.2	2.8	3.8	5.0	6.0	7.2	9.6	10.4
gC-ve	0	1.8	4.6	4.8	6.8	7.0	7.6	8.4	9.6
RFb	0	1.4	3.4	4.2	5.4	6.2	7.0	8.0	9.4

NSC-34 cells									
Time (min)	0	1	2.5	5	10	15	20	30	60
% Adsorbed									
17 ⁺	0	0.2	2.0	3.8	5.2	6.2	7.6	9.8	10.0
gC-ve	0	1.0	1.6	2.2	6.4	6.8	6.8	8.2	8.4
RFb	0	0.6	1.4	2.4	6.0	6.6	7.0	7.8	8.0

Table 2. Titres of Adsorption Studies on BHK, NSC-19, and NSC-34 cells

5.10 *In vitro* Adsorption of RFb

Heparan sulphate moieties of cell surface proteoglycans have been shown to interact with HSV gC in the initial binding of HSV-1 to cells (WuDunn and Spear, 1989). The principal ganglioside ligand of LTB is GM1, however it is also able to weakly bind several other gangliosides (Fukuta *et al.*, 1988). To determine the binding abilities of RFb to heparin and a range of gangliosides a HSV-binding ELISA was carried out as per section 3.39 and 3.40.

Binding of wild type, gC-negative and RFb to heparin *in vitro* mirrored the adsorption of these viruses to BHK cells where heparan sulphate is thought to play a major role in binding of virus (Figure 5.23). Bound virus was detected by a peroxidase-linked antibody complex. Following the addition of substrate solution, optical densities were read at 490nm. RFb and gC-ve both showed a lag in the initial stages of adsorption with respect to wild type, however all virus bound to heparan after 60 min.

Wild type, gC-negative and RFb were then assayed for their ability to bind to a range of gangliosides (section 3.39). Bound virus was detected by spectrophotometer and this was expressed as a percentage of virus bound to heparin after 60 min (Figure 5.24; Table 3).

17⁺ bound to several gangliosides with a higher affinity than to heparin. These included GA1 and GD1b, to which the virus bound slightly stronger and GT1b and GD3 to which the virus bound with a stronger affinity than heparin.

gC-negative HSV-1 bound to GA1 with a similar affinity to heparin, however this binding was not as strong as wild type to this ligand. It also bound less strongly than wild type to several other gangliosides including GD1b, GT1b, GQ1b and GD3, indicating that viral binding to these gangliosides requires gC.

RFb bound the gangliosides in a manner similar to its parental virus gC-negative. It did not bind stronger than gC-negative HSV to gangliosides to which LTB binds i.e. GM1, GM2, GD1b.

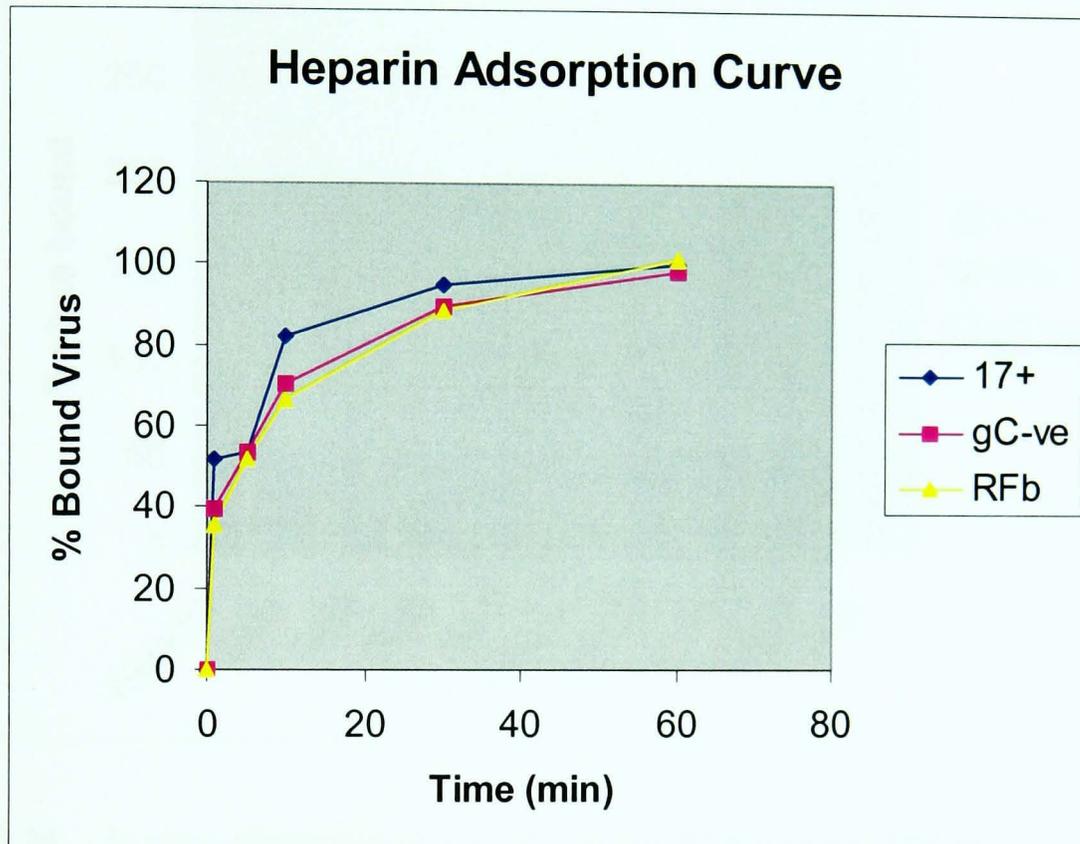


Figure 5.23 *In vivo* adsorption of 17⁺, gC-ve and RFb to Heparin

An ELISA plate was coated with heparin. 1×10^7 pfu/well virus was added to each well. This was incubated at 37°C. At 1, 5, 10, 30 and 60 min unbound virus was removed. Bound virus was detected as described in section 3.39. Results are expressed as a percentage of 17⁺ bound at 60 min. Each time point is the average of three readings.

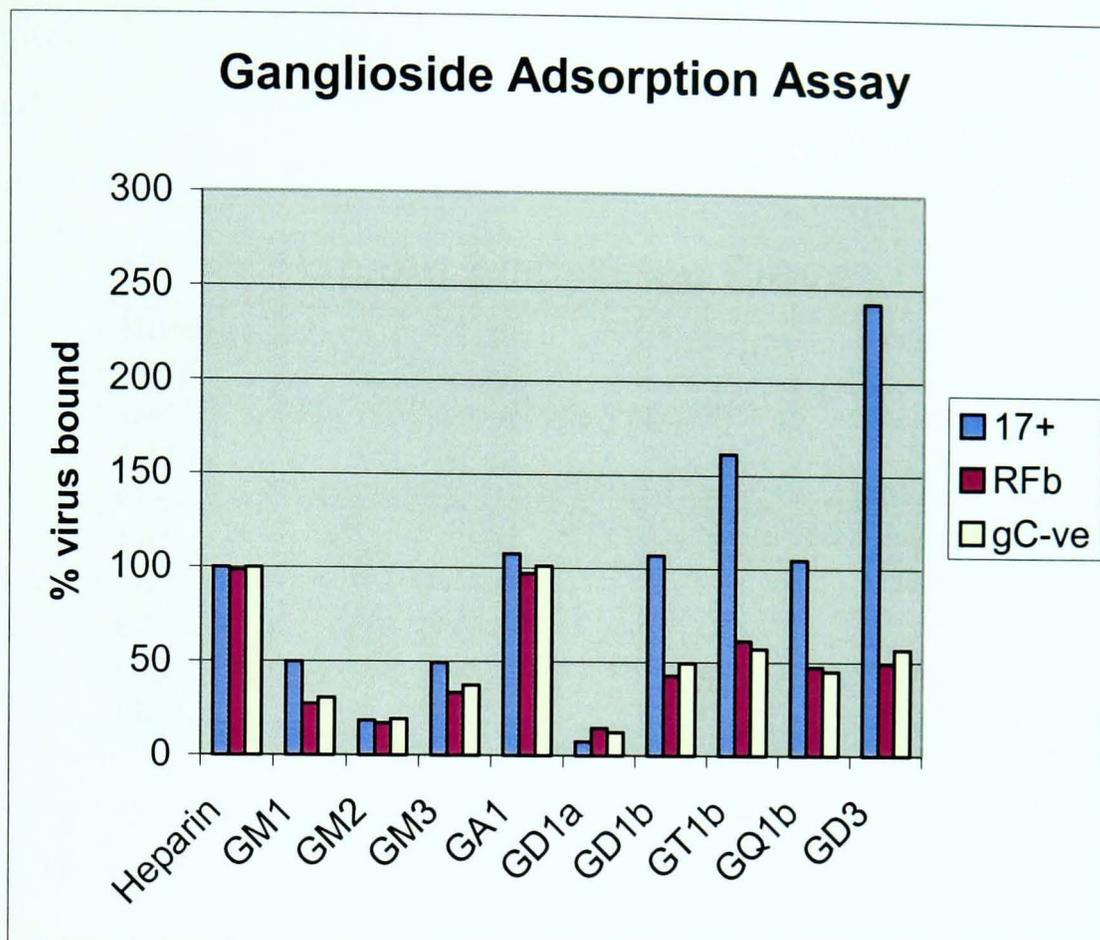


Figure 5.24 *In vitro* adsorption of 17+, gC-ve and RFb to Gangliosides

An ELISA plate was coated with various gangliosides. 1×10^7 pfu/well virus was added to each well and incubated at 37°C for 60 min. Unbound virus was removed by washing and bound virus detected as section 3.39. Bound virus was expressed as a percentage of the respective virus bound to heparin. Each time point is the average of three readings.

	17+	RFb	gC-ve
Heparin	100.00	98.56	100.00
GM1	49.82	27.36	30.57
GM2	18.59	17.08	19.32
GM3	49.47	33.45	37.52
GA1	107.71	97.21	101.24
GD1 _a	7.36	14.71	12.53
GD1 _b	107.01	42.91	49.67
GT1 _b	161.40	61.54	57.21
GQ1 _b	104.91	47.49	45.21
GD3	242.10	49.58	56.82

Table 3. % normalised binding with respect to heparin binding

5.11 Conclusion

Following the purification of RFb and the partial purification of RFa, the properties of these viruses were determined through gene expression, growth characteristics on a variety of cell lines, and adsorption to a variety of cell ligands *in vivo* and *in vitro*.

Initially it was shown that these viruses, like the parental virus, failed to produce glycoprotein C. As a result the mutant viruses had their major HS-binding glycoprotein eliminated. The gene fusion was inserted in the RL1 locus and expression of this gene product, ICP34.5, was investigated by Western blotting. RFb failed to produce ICP34.5, and so would be non-neurovirulent if used in animal studies.

Detection of the LTB-gC fusion protein was ascertained by a number of methods:

Western blotting of infected cell extracts; indirect immunofluorescence of infected cells; transfection of plasmids pRFa and pRFb with subsequent detection by Western blotting and indirect immunofluorescence; concentration of protein extracts prior to Western blotting. However, no fusion protein was detected. There are many possible reasons for this.

Ruddock *et al.*, (1995) have shown that fusion proteins containing LTB can alter the conformation and stability of LTB. By fusing truncated portions of HSV-1 gC to LTB, slight changes in the LTB tertiary structure may be occurring. These could mask the epitopes that antibodies against LTB recognise and so fusion proteins would go undetected by these monoclonals. Anti-gC antisera also failed to recognise any fusion protein in Western blots. Again this may be simply due to the fact that the truncated gC genes encode the cytoplasmic, transmembrane, and in the case of RFa, a small stretch of the extracellular domain. These may not be recognised by the anti-gC antisera. There are several examples of fusion proteins involving LTB which are detectable by anti-LTB antibodies, however, the majority of these are small peptides fused to the B-subunit e.g. a 12 amino acid-peptide linked to LTB (Sandkvist *et al.*, 1987), much smaller than the fusions presented in this work.

Alternatively, the antibodies may not be able to detect the fusion proteins as their level of expression and incorporation into the virus envelope is quite low. In previous work, Laquerre *et al.*, (1998a) constructed fusions of truncated forms of gC and full-length erythropoietin hormone (EPO). One of the fusions contained the cytoplasmic, transmembrane, and part of the extracellular domain (amino acids 376-511) - the same gC truncation that RFa contains. This was poorly incorporated into the viral envelope, approximately fivefold less than wild type gC. RFb contains a more truncated gC in its fusion, and the external gC domain deleted may be required for efficient glycoprotein incorporation into the virus. If this is the case it can be assumed that it would incorporate poorly into the viral envelope, if at all. This would result in very low levels of protein expression and may be too low for the MAbs against LTB to detect. Also due to the size of the gC truncation (cytoplasmic and transmembrane domains remaining) the fusion protein of LTB fused to gC may be sterically hindered from forming a pentamer due to its proximity to the viral envelope.

The mutant virus RFa could not be purified. Following co-transfection, mutant viral plaque isolates containing the insert were detected by Southern blotting. These were found to be contaminated with parental virus. The virus was taken through rounds of purification to obtain a pure virus stock. After the first round of purification the amount of mutant virus had lessened, and following a further round of purification the mutant was lost. The inability of this mutant to be purified may be explained by several ways.

Our experiments using RFa (Western blot analysis of infected cellular extracts, indirect immunofluorescence) used an impure viral plaque isolate from the first round of purification (the step immediately before the mutant virus was lost). If our fusion protein incorporated with 20% efficiency of wild type gC, as the equivalent fusion of Laquerre *et al.*, (1998c) incorporated, then this may have caused poor levels of attachment and/or entry. Thus, if the initial plaque isolate was partially pure, each round of purification would have lessened the amount of mutant virus present until the mutant was diluted out. Also as a consequence there would be very little fusion protein produced which could be detected in these experiments.

From the studies of Stannard *et al.*, (1987) gC appeared to consist of “randomly distributed” long, thin components which extend approximately 20nm from the envelope. Therefore, by replacing 375 residues of gC with 102 residues of LTB, this could result in a more compact conformation of the glycoprotein. This could have the effect of burying the molecule into the envelope and prevent efficient binding, and consequently cause dilution of the virus during purification. As the exact mechanism of binding and penetration is not fully understood, it is also possible that receptor binding physically altered the envelope in a manner that prevented it from carrying out efficient attachment, entry or cell-to-cell fusion.

Campadelli-Fiume *et al.*, (1988a) reported a BHK cell line that expressed HSV-1 gD (BJ-o cells). This cell line was seen to spontaneously fuse causing polykaryocytes. They found that the characteristics and requirements of cell fusion of BJ-o cells are the same for HSV-1 induced fusion. They showed that fusion of cells was greatest when the terminal sialic acid of cellular carbohydrate moieties was available and exposed. This is consistent with earlier reports that HSV-induced cell fusion is reduced in cells treated with neuraminidase, which removes terminal sialic acid residues (Campadelli-Fiume *et al.*, 1988b). LTB binds ganglioside GM1, and less strongly to GD_{1b} (Holmgren *et al.*, 1973) and GM2 (Fukuta *et al.*, 1988). From crystallography studies (Merritt *et al.*, 1994) the binding of LTB to GM1 is described as a “two-fingered grip” in which the Gal-GalNAc “forefinger” of the oligosaccharide is buried deep within the toxin, and the sialic acid “thumb” lies along the toxin surface. GM1, GD_{1b}, and GM2 all contain free sialic acid residues (Figure 5.25).

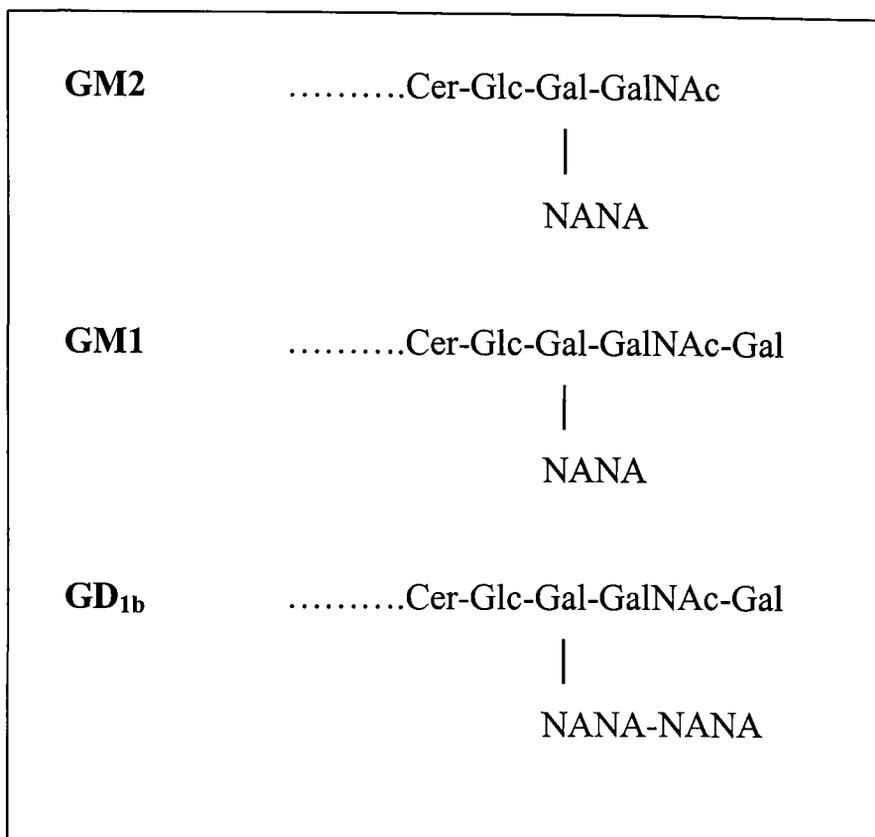


Figure 5.25 Ganglioside structures

Legend: Cer -ceramide; Glc -glucose ; Gal -galactose ; GalNAc -*N*-acetylgalactosamine; NANA -*N*-acetylneuraminic acid (sialic acid)

Therefore, LTB-gC produced by RFa could sequester cellular sialic acids necessary for viral-cell fusion. If low levels of the fusion protein were produced, or if they ineffectively bound to sialic acid this could explain why the virus became diluted and eventually lost during purification.

In vitro transcription of the LTB.gC(b) gene fusion from plasmid pGEM.LTB.gC(b) was carried out using the Ambion MEGAscript T7 transcription kit. This plasmid was chosen as its sequencing showed the cloning of the gene fusion to be error-free (data not shown) and also this plasmid contains the T7 promoter directly upstream of the gene fusion. Following *in vitro* transcription, mRNA of the correct size was seen, showing that this construct was correctly transcribed.

Despite the fact we were unable to identify fusion protein from either virus, further experiments were carried out to characterize them. Replication experiments were carried out in a variety of cell lines.

In BHK cells RFb grew similar to its parental virus gC-negative. The virus initially grew more slowly than wild type, however rates of growth and final titres were similar between the two. In 3T6 cells, a cell line not permissive for RL1-negative mutants, RFb grew poorly, and its growth pattern mirrored that of 1716, a RL1-deleted virus, showing that the gene insert had knocked out RL1 function *in vivo*. In the NSC cell lines, all viruses showed poor growth characteristics. This may be in part due to the morphology of the cells, in that there is poor contact between neighbouring cells, resulting in poor cell-to-cell spread.

The ability of RFb to adsorb to cells *in vivo* was investigated. Normally adsorption experiments are carried out at 4°C. This is because most viruses can bind but are unable to enter cells at this temperature. However, due to the sensitivity of the NSC cell lines to prolonged exposure at 4°C all adsorption experiments were carried out at 37°C. All viruses bound similarly to BHK cells with over 75% of virus binding within the first 10 min. RFb showed a slight lag in adsorption compared to 17⁺ in the first 30 min, however all virus was bound after 60 min. Viruses bound poorly to both NSC-19 and NSC-34, with only 10% of virus binding. In both cell lines wild type virus bound slightly better than RFb or gC-negative. This may be due to the expression of cell ligands on the surface of the cells e.g. both cell lines were shown to be heterogenous for expression of cholera toxin-binding ligands (data not shown); thus only subpopulations of cells may express ligands to which HSV-1 binds e.g. HS, HveA etc. The greater ability of wild type virus to adsorb may be due to the part gC plays in binding NSC-19 and NSC-34 cells.

HSV-binding ELISAs were then carried out to see how virus bound to heparan (a known HSV ligand) and a variety of gangliosides. Binding to heparan mirrored the adsorption seen in BHKs *in vivo*. This is not surprising as BHKs use heparan sulphate as the major

HSV-binding ligand. Initially Rfb bound less heparin, however all virus bound after 60 min. When comparing how Rfb bound to gangliosides it was seen that it did not exhibit any higher degree of affinity to GM1, GM2, or GD_{1b} (gangliosides that LTB binds), in comparison to wild type or gC-negative virus. This lack of binding may be due to poor incorporation of gC-LTB into the envelope, or due to its small size that it was hindered in binding gangliosides. Of note were the gangliosides that 17⁺ bound to a greater extent than heparin. These included GA1, GD_{1b}, GT_{1b}, GQ_{1b}, and GD3. As gangliosides are expressed in many tissues of the nervous system, these may act as ligands for HSV in the human body. 17⁺ bound to GD_{1b}, GT_{1b}, GQ_{1b}, and GD3 in much greater numbers than did Rfb or gC-negative virus, indicating that gC is important in the binding of these gangliosides, at least *in vitro*.

6. FINAL CONCLUSION & DISCUSSION

Over the past decade the concept of gene therapy for human disease has become widely accepted by the medical and scientific community. This concept of curing disease at the genetic level is one of the most radical in recent years, and if successful may prove to be one of the milestones in medical history. Disorders of the CNS and other neurological defects were not prominent candidates in early discussions of gene therapy. Problems arising from the complexity of neuronal interactions, the perceived physical inaccessibility of the CNS, and physiological barriers to the introduction of gene transfer vectors through the blood-brain barrier combined to make gene therapy seem less feasible in the nervous system than other organs. However, in recent years the genetic components of many genetic diseases have been identified e.g. Alzheimer's disease (Mullan, 1992), amyotrophic lateral sclerosis (Rosen *et al.*, 1993), making nervous system disorders a target for gene therapy. The aim of researchers now is to develop safe methods of delivering therapeutic genes to target organs, ensuring tissue-specific and regulatable gene expression. This development of vector systems for the direct transfer of genes *in vivo* will be crucial to the development of this medical strategy.

Attenuated or replication-defective recombinant viruses carrying therapeutic genes represent attractive candidates for *in vivo* gene delivery. These can utilise cell surface receptors to gain entry to the cell, where transgene expression can take place. Many viruses including HSV are capable of persisting in a non-pathogenic, latent state requiring the expression of few gene products for maintenance of latency. Eliminating pathogenic virus properties while retaining the ability of the virus to establish latency or a latent-like state provides the core strategy for long-term gene therapy using viral vectors.

Herpes simplex virus has many features that make it a suitable candidate for gene therapy to neuronal cells:

- HSV-1 infects postmitotic neurons and can be maintained indefinitely in a latent state (Stevens, 1975)
- Latent HSV-1 is quiescent: HSV-1 gene expression is limited at most to the IE genes and a latency-associated transcript (Wagner *et al.*, 1988); DNA replication does not occur (Stevens, 1975); No progeny virus are produced (Stevens, 1975)
- In its latent state a neuronally active latency-specific promoter system can be used to express transgenes (Fink *et al.*, 1996)
- The genetics of HSV are well studied and the virus genome can be manipulated to accept large inserts of foreign DNA (Knipe *et al.*, 1978)

The cytotoxic features of HSV have been eliminated by the deletion of immediate early (IE) genes, which prevent the expression of both early and late genes. As a result virus replication or reactivation from latency can be achieved without viral replication (Dobson *et al.*, 1990). Another approach is the deletion of genes required specifically for growth in neuronal cells, but not in other cell types. This latter method has the advantage that virus does not need to be grown up on cell lines expressing the essential genes which allows for the possibility of regeneration of wild type virus by recombination.

HSV-1 has a wide host range, infecting many cell types. To target HSV vectors to a specific cell type, the natural tropism of wild type virus will have to be eliminated, or at least altered, and vectors may have to express new ligands which are capable of binding cell surface receptors on the target cell surface, without affecting cell penetration or cell-to-cell spread. gC is the principal glycoprotein involved in binding of HSV to HS, however it is non-essential and can be deleted. To target HSV-1 to motor neurons, the HS-binding domain was deleted and replaced by *E. coli* heat-labile enterotoxin B subunit (LTB). LTB is a non-toxic protein which forms pentamers that bind several gangliosides including GM1, GD_{1b}, GM2 and asialo-GM1. Gangliosides are complex glycolipids found at high levels in neuronal cells (Wiegandt, 1985), with GM2 thought to be a major ganglioside in motor neurons (Yoshino *et al.*, 1994). Thus it was hoped that this novel glycoprotein could alter the tropism from sensory neurons, towards motor neurons.

In this work, experiments were designed to meet several objectives:

- (i) Construct gene fusions between LTB and varying lengths of truncated gC
- (ii) Construct viruses containing these fusions that are non-neurovirulent and have their natural binding property eliminated
- (iii) Analyse the gene expression of these viruses to ensure correct production of the fusion proteins
- (iv) Analyse the binding capabilities of these viruses to determine if the chimeric molecules have novel binding function
- (v) Analyse by β -Gal staining *in vivo* using mouse models, that the mutant viruses had altered their tropism towards motor neurons

Chimeric gene fusions were constructed between LTB and two differing lengths of gC. These were sequenced to ensure that the fusions were in-frame. They were combined into a gC-negative LacZ virus at the RL1 locus. Mutant virus RFb was constructed and shown to be both gC-negative and ICP34.5-negative, thus achieving the first aims of the experiment. Virus RFa failed to be constructed. Positive plaque isolates were discovered following co-transfection, however, the virus failed to be purified. Several reasons were suggested for this. Laquerre *et al.*, (1998c) produced a similar fusion of gC with the hormone EPO. This was found to have incorporated into the viral envelope fivefold less than wild type gC. If our fusion inserted into the envelope at a similar level this may have caused poor levels of attachment and/or entry. Alternatively due to the size of the construct (237 LTB-gC amino acid residues as opposed to 511 gC amino acid residues) the construct may have become buried deep within the viral envelope, again causing inefficient binding. If however, binding of the fusion protein did take place, the possibility that receptor binding physically altered the envelope in a manner that prevented it from carrying out efficient attachment, entry or cell-to-cell fusion cannot be ruled out. Finally Campadelli-Fiume *et al.*, (1988(a)(b)) reported that HSV-induced cell fusion is reduced in cells treated with neuraminidase, which removes terminal sialic acid residues. LTB binds a variety of gangliosides, and studies of its binding have showed that

it requires a free sialic acid to bind. Thus, the fusion protein may have bound this, sequestering cellular sialic acids necessary for viral-cell fusion.

Mutant virus RFb was analyzed to determine if it expressed the fusion protein in a form that could bind ligands. No fusion protein was detected by Western blot or indirect immunofluorescence. This may have been caused by conformational changes in LTB brought about by fusion to gC which masked epitopes recognized by anti-LTB MAbs. Alternatively, the level of protein produced may have been too low to be recognized by the MAbs. Laquerre *et al.*, (1998(b)) showed that modification of a HSV-1 glycoprotein can result in defective recombination molecule processing and/or intracellular trafficking and subsequent failure to incorporate the modified glycoprotein into the virion envelope. The possibility that this occurred with LTB-gCb cannot be ruled out. A further possibility is that the gene fusion may not have been transcribed or translated correctly, however, *in vitro* transcription of the LTB.gC(b) gene fusion showed the presence of LTB-gC mRNA. Sequencing of the expression plasmids pRFa and pRFb was carried out and no mutations were detected in the gene fusions. The CMV IE promoter was chosen to drive expression of the fusion as this was a non-HSV promoter and so would lessen the chances of recombination occurring at an incorrect position in the genome. This has been shown to generate high levels of expression however, as the fusion was inserted into the RL1 locus, the use of the RL1 promoter may have been more pertinent, however several recombinant viruses have been constructed with the CMV IE promoter at the RL1 locus with excellent expression of the transgene (S.M. Brown, personal communication).

Despite being unable to detect the novel chimeric protein, mutant virus RFb was characterized. Its replication in several cell lines showed that it grew similar to gC-negative HSV-1 in BHK cells, while the fact that it had RL1 deleted meant it failed to grow in 3T6 cells. In the motor neuron-like cell lines (NSC-19 and NSC-34) where it was hoped the virus may have grown better due to its LTB-containing glycoprotein, no discernable difference in replication characteristics were distinguished between it and its parental virus. Binding assays carried out on BHK, NSC-19 and NSC-34 cells also failed to show any increase in the rate of adsorption with respect to its parental virus. Finally to investigate whether the virus was perhaps binding more efficiently to the motor neuron

cells, but was not seen due to some problem with viral entry, a HSV-1 binding ELISA to several gangliosides was carried out. A heparin-binding ELISA was also carried out and results of the ganglioside assay were expressed as a percentage of heparin binding. Increased binding to several gangliosides known to bind LTB was not seen in RFb, with binding mirroring that of gC-negative virus. 17⁺ however, bound several gangliosides to a greater extent than heparin. These included GA1, GD_{1b}, GT_{1b}, GQ_{1b}, and GD3. As several of these are expressed in the human nervous system, these may be natural HSV-1 binding ligands *in vivo*. 17⁺ also bound GD_{1b}, GT_{1b}, GQ_{1b}, and GD3 to a much higher degree than either of the gC-deleted viruses. Therefore it can be postulated that gC plays a key role in binding these gangliosides. Binding of wild type virus to ganglioside GD3 was especially high. This ganglioside is a major glycolipid component of the developing central nervous system but diminishes considerably as the CNS matures (Goldman and Reynolds, 1996). In the adult CNS GD3 is expressed in low amounts by some neuronal subpopulations, on reactive and resting microglia, and by reactive astrocytes (Goldman and Reynolds, 1996). Much of the information we have about HSV-1 adsorption/penetration has been determined from non-neuronal tissue e.g. epithelial cells and HS is seen to be the major cellular ligand involved in initial binding of virus to cell. Thus, it may be *in vivo* that GD3 acts as the major ligand for HSV-1. This may be important as administering anti-GD3 antibody could potentially block the entry of HSV-1 into neurons.

As RFb did not exhibit any signs of novel binding towards gangliosides, it was decided not to investigate the effectiveness of this virus in an animal model.

While many of the objectives of the experiments were not achieved, the idea of altering the tropism of HSV-1 to act as a gene therapy vector is still one worth investigating. There are several possible ideas for future work to be carried out on this project.

To avoid steric hindrances between the viral envelope and the fusion protein, fusions involving less truncated portions of gC could be investigated. Possible truncations could include amino acid residues 280 to 511, or 120 to 511, as both these truncations contain

cysteine residues known to form disulphide bonds, which may be of importance in maintaining the tertiary structure of gC. These should therefore place the LTB protein further from the viral envelope facilitating ganglioside binding.

While LTB has many characteristics that make it a suitable candidate as a binding ligand to motor neurons, namely its stability and wide binding range, it also has many drawbacks. These include the fact that LTB must pentamerise to become a functional binding protein. As our knowledge of the organization of glycoproteins in the viral envelope and interactions between them is superficial, the ability of a gC-LTB chimera to pentamerise may prove difficult. This may cause subsequent problems with other glycoproteins, which in turn may affect various aspects of viral growth, namely viral fusion, entry and cell-to-cell spread. Thus it may be more suitable to construct vectors which contain binding ligands that do not need oligomerization prior to binding.

Fusion proteins of HSV glycoproteins with binding ligands seem logical to ensure their incorporation into the viral envelope, as it is unknown whether glycoprotein incorporation requires specific signals or interactions. This idea is supported by Anderson *et al.* (2000), who found that vesicular stomatitis virus G protein was incorporated into HSV-1 virions but the efficiency of incorporation was increased if the transmembrane domain was replaced with that of HSV-1 gD. However, a paper by Whiteley *et al.* (1999) showed that the transmembrane of gD can be replaced by the corresponding domain of 2-sialyl transferase without a discernable decrease in gD incorporation or virion infectivity. This throws into doubt the idea of HSV-glycoprotein interactions prior to incorporation in the viral envelope. Also Dolter *et al.* (1993) reported the incorporation of human CD4 receptor into HSV-1 albeit at poor levels of incorporation. Taken together, this suggests that a novel receptor may be incorporated into a HSV vector without prior modifications, thus ensuring its native conformation and maximal receptor function, however gene fusions with some HSV glycoprotein genes may increase its level of incorporation.

A further drawback of using LTB in future viral vectors is its characteristic as a powerful immunogen. LTB can function as carriers for mucosal delivery of vaccine antigens

(Schodel and Will, 1989; Nashar *et al.*, 1993) and can possess some adjuvant activity (Verweji *et al.*, 1998). It is unknown whether this immunogenic effect of LTB could hinder the progress of the vector if it was used in gene therapy *in vivo*. However, replacement of LTB with a less-immunogenic protein may ameliorate this problem.

A consequence of this immunogenic-role is that while the vector may elicit a high immune response that may hinder its success in gene therapy, the vector may become an excellent vaccine against HSV. HSV-1, while ubiquitous in the human population is the leading cause of acute sporadic fatal viral encephalitis in the United States and is responsible for over 200,000 cases of blindness every year (Fresney, 1983). Because of the location of glycoproteins on the surface of infected cells, they act as major antigenic determinants for the cellular and humoral immune responses of the host (Norrild, 1985; Spear, 1985). gC binds the C3b fragment of the third component of complement (Friedman *et al.*, 1984). This region has been located to Cys-1 (aa 127) and Cys-2 (aa 144), which forms a disulphide bond, producing a loop which forms a binding region. Thus, genetic fusion of gC and LTB may provoke an excellent immune response when administered.

6.1 Position of research approach relative to gene therapy strategies for neurological disease

Since commencement of the first human gene therapy trial in 1989, the field has grown to more than 400 currently approved trials (Hsich *et al.*, 2002). Only a few, however, have been directed to diseases of the nervous system, including treatment of brain tumours and degenerative diseases (Rampling *et al.*, 2000; Anderson, 2000; Tuszynski *et al.*, 2002). The lack of more trials is due in part to the difficulties in designing positive interventions in the nervous system, a system so complex and critical to human integrity. Early concerns focussed on inherent toxicity and immunogenicity of vectors, as mild inflammatory responses and edema can cause damage to neurons, and immune responses to self-antigens can be elicited. Other damaging responses to injection injury include hemorrhage, infection and gliosis, which can lead to epileptic foci (Albensi, 2001).

Further, delivery constraints within the brain are formidable, with focal injections usually limited by the rapid cellular uptake of vectors and their relatively large size (20-150nm), which restricts diffusion within the brain parenchyma, as well as the need to use small volumes and slow delivery times to avoid compression damage. Attempts at more global brain delivery via the cerebrospinal fluid and vasculature were initially frustrated by high toxicity of ventricular injections and difficulties in breaching the blood-brain barrier.

The most important issue today in the development of gene therapy vectors is the potential risk to patients, as highlighted by the death of an 18-year-old patient following a hepatic-arterial infusion of a replication-defective adenovirus vector during a gene therapy trial for ornithine transcarbamylase deficiency (Somia and Verma, 2000). This death was due to a massive cytokine response to the adenoviral vector vector, resulting in disseminated intravascular coagulation (Schnell *et al.*, 2001). Several safety issues exist today:

Vector/delivery toxicity

- Vector may be directly toxic to cells e.g. through low-level expression of viral proteins
- Vector or transgene products may cause an inflammatory response, and may facilitate an immune reaction to protein in deficient patients, such that they can no longer receive protein replacement therapy, or may cause autoimmune reaction
- Route of delivery may be damaging e.g. injection of the vector into the spinal cord may cause mechanical damage
- Transgene product may have untoward effects e.g. growth factors may cause sprouting and misconnection of neurons
- Replication-conditional vectors may cause damage to normal tissue via inflammation and cell death and may activate latent viruses

Gene delivery to the CNS is complicated by difficulties in access, which usually require neurosurgical procedures, and the need, in some cases, for disseminated delivery over extended regions. Direct intraparenchymal injections are limited by the small volumes

that can be injected into focal areas. Diffusion is minimal in nervous tissue, so cells at the injection site may take up a large number of virions whereas those over a few micrometres to millimetres away may not be transduced. Many vectors can move by retrograde or anterograde transport within neuronal processes, however, and thus traverse extensive distances. Further, neuronal processes can serve to spread transgene products e.g. retinal neurons infected in the eye with AAV vectors project back through the optic tract and release corrective lysosomal enzyme to the brain proper (Hennig *et al.*, 2001).

A promising strategy for global or directed gene delivery has been the use of *ex vivo*-modified migratory cells, such as neuroprogenitors, which can migrate extensively in the damaged adult brain and can be used as biologic sources of therapeutic gene products or as carriers of replication-competent vectors (Herrlinger *et al.*, 2000) or on-site retrovirus production (Lynch *et al.*, 1999; Sena-Esteves *et al.*, 1999). Global delivery can also be achieved through the vasculature by temporary disruption of the blood-brain barrier (BBB). Virus vectors can pass across the BBB, and more easily the blood-tumour barrier, with temporary vascular disruption by pharmacologic or osmotic agents (Nilaver *et al.*, 1995; Rainov *et al.*, 1995); by the use of cells, such as endothelial cells, which can migrate across the vasculature (Messina *et al.*, 1992); or by targeting to the transferrin receptor, which can transport compounds across the microcapillary endothelium (Xia *et al.*, 2000; Bickel *et al.*, 2001). However, the BBB provides protection for the brain, and breach of this barrier can have toxic consequences through fluid influx leading to edema, changes in electrolyte balance, and access to blood-borne pathogens (Inamura and Black, 1994).

Thus, the experimental approach conducted in this thesis has a valid place in the development of effective viral gene therapy vectors for the treatment of neurological disease. Phase one clinical studies of RL1-null HSV-1 vectors in the treatment of brain tumours (Rampling *et al.*, 2000; Markert *et al.*, 2000) were successful, showing this vector system to be potentially safe in humans. While some concerns may exist regarding the safety of the virus, e.g. recombination with latent wild type virus, the many attributes of HSV-1 make it an exciting candidate for further studies. Also, by directing the virus to

the motor neuron system via injection of the gastrocnemius muscle potential damage to the system is averted e.g. in comparison to direct injection. If future vectors can be directed efficiently to the motor nervous system, then the ability of the virus to remain latent while expressing exogenous therapeutic genes through the LAT promoter, could provide the solution to many neurological diseases.

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