

**HERPES SIMPLEX VIRUS VECTORS FOR GENE  
DELIVERY TO THE CNS: APPLICATIONS IN  
THE STUDY OF ALZHEIMER'S DISEASE**

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## Abstract

Understanding the interactions between the presenilins and amyloid precursor protein (APP) remains one of the most challenging questions in Alzheimer's research. *In vivo* studies are currently limited to the introduction of exogenous genes into the germline to produce transgenic mice. The study of gene combinations then requires the generation and subsequent crossing of multiple transgenic lines.

This thesis reports the development of a system to offer improvements to both cell culture and animal studies of APP/presenilin interactions. This system is based on disabled herpes simplex virus vectors which can directly deliver genes to neurons either *in vitro* or *in vivo*, allowing multiple mutations to be rapidly introduced and evaluated. The viral vectors constructed have been disabled by the deletion or inactivation of genes encoding ICP34.5/ORF P, VP16, ICP27, ICP4 and *vh*s and consequently do not express significant amounts of any of the IE gene products. The vectors are non-toxic to cultured neurons at high MOI and are capable of expressing multiple exogenous genes in cell culture or *in vivo*. Gene expression is maintained in the long term using promoter systems containing elements from the HSV1 latency associated transcript promoter. Following injection into the CNS *in vivo*, widespread exogenous gene expression occurs as a result of retrograde transport of the vector from the site of inoculation to connected sites within the nervous system.

Wild type and mutant APP and presenilin 1 were introduced into these viruses both individually and in combination. The viruses were then used to deliver these genes to primary neuronal cultures from rodent and non-rodent origins. The significance of using non-rodent neurons and observations from these experiments are described. The HSV-1 system described in this thesis should provide powerful means by which gene combinations relevant to Alzheimer's disease can be rapidly introduced and evaluated.

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## **Declaration**

All the work presented in this thesis is the work of Caroline Lilley. Contributions by other researchers are acknowledged below:

1. Human fetal neurons were prepared by Ritchie Williamson, Institute of Psychiatry, King's College London.
2. Embryonic cortical neurons (mouse and rat) were prepared by Liz Ensor, Institute of Child Health, University College London.
3. Adult rat DRG neurons were prepared by James Palmer, Department of Molecular Pathology, University College London.
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6. The caspase assays described in chapter 8 were carried out by Tim Allsopp, University of Edinburgh.

## **Publications**

Some of the results arising from this thesis are the subject of research communications:

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Palmer, J., Branston, R., **Lilley, C.E.**, Robinson, M., Groutsi, F., Smith, J., Latchman, D.S. and Coffin, R.S. (2000) Development and optimisation of herpes simplex virus vectors for multiple long-term gene delivery to the peripheral nervous system. *J. Virol.* **74**, 5604-5618

Thomas, S.K.†, **Lilley, C.E.†**, Latchman, D.S., and Coffin, R.S.(1999). Equine herpesvirus 1 gene 12 can substitute for vmw65 in the growth of herpes simplex virus (HSV) type 1, allowing the generation of optimized cell lines for the propagation of HSV vectors with multiple immediate- early gene defects. *J Virol* **73**, 7399-7409.

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Wagstaff, M.J., **Lilley, C.E.**, Smith, J., Robinson, M.J., Coffin, R.S., and Latchman, D.S.(1998). Gene transfer using a disabled herpes virus vector containing the EMCV IRES allows multiple gene expression in vitro and in vivo. *Gene Ther* **5**, 1566-1570.

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Coffin, R.S., Latchman, D.S., **Lilley, C.E** , Thomas, S.K., Branston R., Palmer, J. (2000) Herpes simplex virus vectors for neurological applications in *Targetting of drugs: strategies for gene constructs and delivery*. (Nato Science Series). Edited by

Gregory Gregoriadis and Brenda McCormack pp19-33. Published by IOS Press, Amsterdam.

These papers are appended to the back of this thesis.

## **Abstracts**

**Lilley, C.E.**, Groutsi, F. Han, Z., Anderson, P.N., Latchman D.S. and Coffin, R.S. (2000) Multiply disabled herpes simplex virus vectors for gene delivery to the central nervous system. In press, *Molecular Therapy* May 2000. (Journal of the American Society for Gene Therapy)

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Wright, M.J., Wightman, L., **Lilley, C.E.**, de Alwis M., Coffin, R.S., Latchman, D.S., Thrasher, A., Marber, M.S. Differences in time course of gene expression following direct intramyocardial injection of recombinant adenovirus, adeno-associated virus and herpes simplex virus. *Heart* 99, 81 (Supplement 1): 56

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## Abbreviations

A	adenosine
AADC	aromatic amino acid decarboxylase
AAV	adenoassociated virus
A $\beta$	$\beta$ -amyloid peptide
Ad	human adenovirus
APLP	amyloid precursor like protein
APP	amyloid precursor protein
APS	ammonium persulphate
ATP	adenosine triphosphate
BGH	bovine growth hormone
BHK	baby hamster kidney
BHV	bovine herpes virus
bis	n,n'-methylene-bis-acrylamide
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CAR	coxsackievirus and adenovirus receptor
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
CHAPS	3,3 cholamidopropyltrimethylammonio-1 propane sulphonate
CIAP	calf intestinal alkaline phosphatase
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CTP	cytosine triphosphate
d	deoxy
dd	dideoxy
ddH <sub>2</sub> O	double distilled water

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ds	double stranded
E	early (class of genes)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol bis (beta-aminoethyl ether)-N,N'-tetracetic acid
EHV	equine herpes virus
ELISA	enzyme-linked immunosorbant assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
FCS	fetal calf serum
g	gram
G	guanosine
gB	glycoprotein B (similarly for other glycoproteins)
GDNF	glial derived neurotrophic factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HCF	host cell factor
HEK	human embryonic kidney
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HIV	human immunodeficiency virus
HMBA	hexamethylene bisacetamide
HMG-1	high mobility group protein 1
HRP	horseradish peroxidase
HSDNA	herring sperm DNA
HSV1	herpes simplex virus type 1
HVEM	herpes virus entry mediator

IAA	isoamyl alcohol
ICP	infected cell polypeptide
IE	immediate early (class of genes)
IL	interleukin
IRES	internal ribosome entry site
ITR	inverted terminal repeat
kb	kilobase
kDa	kilo Dalton
l	litre
L	late (class of genes)
LAP	latency active promoter
LAT	latency associated transcript
LB	Luria Bertani medium
LMP	low melting point
LTR	long terminal repeat
M	molar
mA	milliamps
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
MMLV	Moloney murine leukaemia virus
MMTV	mouse mammary tumour virus
MOI	multiplicity of infection
mRNA	messenger RNA
MSV	murine sarcoma virus
MW	molecular weight
ng	nanogram
NGF	nerve growth factor

nm	nanometre
NP40	nonidet P40
nt	nucleotide
NTP	nucleotide triphosphate
Oct	Octomer
OD	optical density
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS with 0.1% tween-20
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post infection
PMSF	phenylmethanesulphonyl fluoride
PNS	peripheral nervous system
PS1	presenilin 1
PS2	presenilin 2
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT	room temperature
SDS	sodium dodecyl sulphate
ss	single stranded
SSC	standard sodium citrate
SV40	simian virus 40
T	thymidine
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TBP	TATA binding protein



TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	transcription factor
TH	tyrosine hydroxylase
Tris	tris(hydroxyl)aminomethane
TTP	thymidine triphosphate
Tween-20	polyoxyethylene-sorbitan monolaurate
V	volt
vhs	virion host shut off
VP	virion protein
v/v	volume for volume
VZV	varicella zoster virus
WT	wild type
w/v	weight for volume
X-Gal	4-chloro, 5-bromo, 3-indolyl- $\beta$ -galactosidase
$\mu$ g	microgram
$\mu$ l	microlitre

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# **CHAPTER 1**

## **INTRODUCTION**

## 1.1 The pathology of Alzheimer's disease

Alzheimer's disease (AD) is a devastating neurodegenerative disorder first recognised by Alois Alzheimer in 1907. AD is now recognised as a common disease of the elderly and is estimated to affect up to 10% of people over 65 years of age and up to 40% of people over 80 years of age. Duration of AD from first symptoms of memory loss until death is approximately 10 years, during which time there is enormous social and economic cost to both patient and society (Albert, 1996).

Diagnosis of AD is usually post-mortem, the classic pathology being the presence of multiple neuritic plaques and neurofibrillary tangles in the brain regions and neural circuits involved in memory and cognition. These are predominantly the neurons of the neocortex, hippocampus, amygdala, basal forebrain cholinergic system and brainstem monoaminergic nuclei (Whitehouse *et al.*, 1982; Morrison and Hof, 1997).

The neuritic plaques are largely extracellular lesions and consist of deposits of a 40 to 42-43 amino acid peptide called  $\beta$ -amyloid ( $A\beta$ ) (Roher *et al.*, 1986).  $A\beta$  species are peptides of approximately 4kDa which result from the processing of amyloid precursor protein (APP), a type 1 single transmembrane glycoprotein (Glenner and Wong, 1984; Kang *et al.*, 1987). The extracellular  $A\beta$  deposits are intimately associated with dystrophic neurites, activated microglia and reactive astrocytes. Approximately 90% of secreted  $A\beta$  peptides are  $A\beta_{40}$ , a soluble form of the peptide. The remaining 10% are  $A\beta_{42}$  and  $A\beta_{43}$ , highly fibrillogenic and readily aggregated species, which are deposited early and selectively in amyloid plaques (Iwatsubo *et al.*, 1994).

The neurofibrillary tangles are intracellular lesions consisting of bundles of straight or paired helical filaments, both of which are predominantly composed of highly phosphorylated forms of the microtubule associated protein, tau ( $\tau$ ) (Grundke-Iqbal *et al.*, 1986a; Grundke-Iqbal *et al.*, 1986b; Goedert *et al.*, 1992). The neurofibrillary lesions are also immunoreactive for ubiquitin, microtubule associated protein 2 (MAP-2) and neurofilament proteins (Anderton *et al.*, 1982; Perry *et al.*, 1987).



## 1.2 The genetics of AD

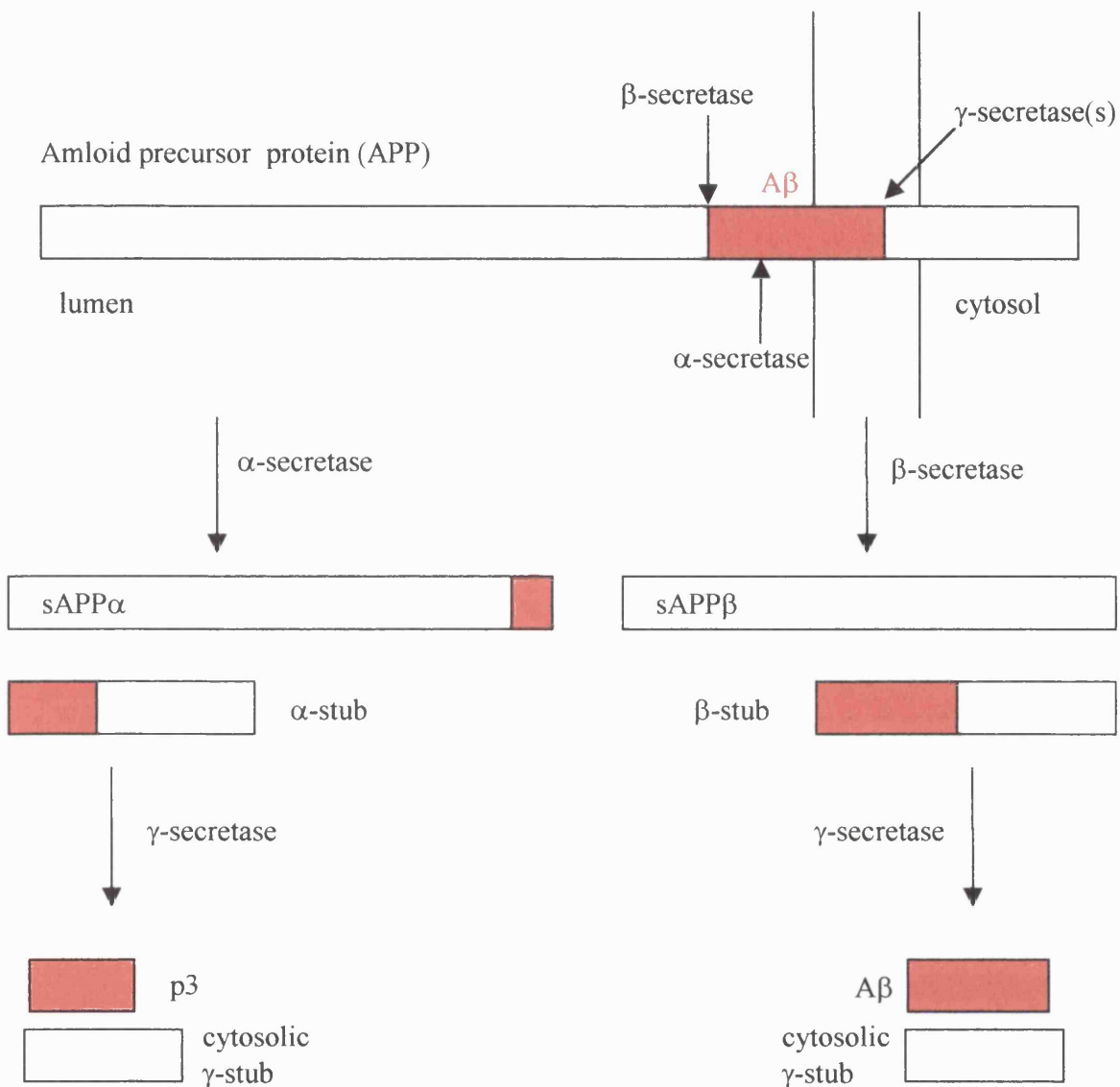
High prevalence and late age of onset are features usually considered uncharacteristic of a genetic disease. However, the recent identification of genes which can cause or modulate AD has clearly demonstrated that this disease can have a genetic basis. The five genes which have been implicated in the pathogenesis of AD are discussed below.

### 1.2.1 Amyloid precursor protein (APP)

APP is a single transmembrane domain glycoprotein which is proteolytically processed to form A $\beta$ . Three major isoforms of APP are produced by alternate splicing. Isoforms of 751 and 770 amino acids are ubiquitously expressed whereas the 695 amino acid isoform is only expressed to high levels in neurons (Koo *et al.*, 1990). The difference between the 751/770 and the 695 amino acid isoforms is that the former include an exon that encodes a serine protease inhibitor domain (Kunitz protease inhibitor or KPI domain) (Tanzi *et al.*, 1988; Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988). The structure of APP is shown in figure 1.2.1.1.

APP species can undergo specific proteolytic cleavages at three sites either immediately flanking or within the A $\beta$  peptide sequence. The importance attributed to the enzymes which mediate these cleavages is indicated by the fact that they were named long before their formal identification. The cleavage sites and resulting proteolytic fragments of these so-called  $\alpha$ ,  $\beta$  and  $\gamma$  secretases are indicated in figure 1.2.1.1. The principal secretory cleavage is effected by  $\alpha$ -secretase, an event which precludes the generation of A $\beta$  (Esch *et al.*, 1990; Sisodia, 1992). The formal identity of the constitutive  $\alpha$ -secretase remains obscure but site directed mutagenesis of APP has suggested that this enzyme has little sequence specificity (Sisodia, 1992). Rather, it cleaves APP at a specific distance from the outer membrane surface to generate a large soluble ectodomain fragment (sAPP $\alpha$ ) and a membrane retained 83 amino acid C-terminal fragment ( $\alpha$ -stub). An alternative pathway involves cleavage by a  $\beta$ -secretase which cuts between residues 671-672 of APP to generate sAPP $\beta$  and a  $\beta$ -stub. The  $\alpha$  and  $\beta$  stubs are then substrates for the  $\gamma$  secretase which cleaves at various sites within a downstream sequence between amino acids 712 and 717 (Haass and Selkoe, 1993). The

reason for this apparent flexibility of  $\gamma$ -secretase cleavage sites is unknown but proposed explanations have included the suggestion that the enzyme has a relaxed target sequence specificity, that there are numerous  $\gamma$ -secretases, or that cleavage initially occurs at the most downstream site and then carboxypeptidase activity removes the C-terminal residues (Tischer and Cordell, 1996; Klafki *et al.*, 1996; Murphy *et al.*, 1999). The  $\gamma$ -secretase cleavage of the  $\alpha$ - or  $\beta$ -stubs generates a small peptide known as p3 or A $\beta$ , respectively. In each case, a cytosolic fragment ( $\gamma$ -stub) is also assumed to be produced although this fragment has never been detected. Over the last five years, numerous candidates have been proposed to be the elusive  $\beta$ -secretase but the formal identification of this enzyme as a novel transmembrane aspartic protease was recently simultaneously reported by four groups (Yan *et al.*, 1999; Vassar *et al.*, 1999; Hussain *et al.*, 1999; Sinha *et al.*, 1999). The identity of the  $\gamma$  secretase(s) remains unresolved and highly controversial. This controversy will be discussed further in section 1.2.2.



**Figure 1.2.1.1 The structure and processing of APP**

The structure of APP and the cleavage sites of  $\alpha$ ,  $\beta$  and  $\gamma$  secretases are indicated. APP is known to undergo proteolytic processing down two pathways. Cleavage by either  $\alpha$  or  $\beta$  secretase occurs first to release a soluble ectodomain and a C-terminal stub. These C-terminal stubs are then cleaved by  $\gamma$  secretase to release A $\beta$  (from the  $\beta$ -stub) or the smaller peptide p3 (from the  $\alpha$ -stub).

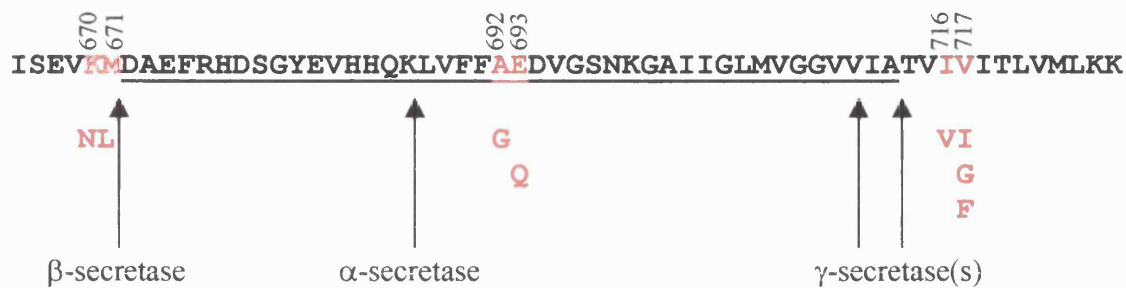
An appealing model for APP processing would be that  $\alpha$ -secretase cleavage was the normal physiological pathway and that cleavages by  $\beta$  and  $\gamma$  secretases were aberrant events which led to the generation of a pathological peptide, A $\beta$  (Sisodia *et al.*, 1990). This simple hypothesis was proved to be incorrect by the observation that *in vitro*, intact A $\beta$  is secreted constitutively by normal cells and *in vivo*, there is A $\beta$  in plasma and cerebrospinal fluid (CSF) of normal individuals (Haass *et al.*, 1992; Shoji *et al.*, 1992; Seubert *et al.*, 1992). It is now well recognised that the cleavages mediated by  $\beta$  and  $\gamma$  secretases occur both physiologically and pathologically to generate the N and C termini of the A $\beta$  peptide, respectively.

Attempts to assign a physiological function to APP appear to have been recently neglected in favour of studying the processing mechanisms of this protein. However, proposed functions include an autocrine factor involved in the stimulation of cell proliferation (Saitoh *et al.*, 1989), mediation of neurite outgrowth in response to nerve growth factor (NGF) (Milward *et al.*, 1992) and numerous roles in cell signalling pathways (Nishimoto *et al.*, 1993; Greenberg *et al.*, 1994). Although the data describing these putative functions are individually convincing, the relevance of these isolated *in vitro* observations to the biological function of APP *in vivo* remains obscure. The physiological role of APP has been further investigated by the creation of homozygous APP-knockout mice (Zheng *et al.*, 1995). Somewhat surprisingly, considering the *in vitro* observations outlined above, these mice proceed through gestation normally and show only minor cerebral gliosis and decreased locomotor activity later in life. This suggested that the APP deficiency may have been at least partially compensated, perhaps by a family of homologous proteins known as the amyloid precursor like proteins (APLPs). Support for this proposed functional redundancy of APP came from the generation of APP/APLP2 knockout mice (von Koch *et al.*, 1997). Approximately 80% of these double knockout mice died shortly after birth and the surviving mice showed deficits in balance, strength and mating ability.

The APP gene is located on chromosome 21q (Goldgaber *et al.*, 1987). Simple overexpression of the APP gene can lead to AD pathology. The significance of this gene dosage effect is demonstrated by the pathology of trisomy 21 (Down's syndrome). In this case it is assumed that a life-long overproduction of both A $\beta$ 40 and A $\beta$ 42 accounts

for the early appearance of amyloid plaques and clinical symptoms of AD observed in these individuals. APP was the first gene in which specific mutations were found to cause familial AD (FAD).

The first pathogenic mutation identified in APP was at codon 693, within the A $\beta$  sequence. This mutation was found in a Dutch kindred with an autosomal dominant hereditary cerebral haemorrhage with amyloid (Levy *et al.*, 1990). This disease is manifested as an adult-onset cerebrovascular disease which was found to result from the deposition of A $\beta$  in the cerebral vessels. Another family with presenile dementia and cerebral haemorrhages were found to have a mutation in codon 692 of APP, also within the A $\beta$  sequence (Hendriks *et al.*, 1992). A second group of pedigrees with early onset FAD were found to harbour mutations which lead to amino acid substitutions at residue 716 or 717, within the transmembrane domain of APP, near the  $\gamma$ -secretase cleavage site (Murrell *et al.*, 1991; Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991). Cells that express APP harbouring the 717 mutations do not appear to secrete higher levels of total A $\beta$ , but secrete an increased ratio of A $\beta$ 42:A $\beta$ 40 relative to cells overexpressing wild type APP (Suzuki *et al.*, 1994). Two large related families from Sweden with early-onset FAD were found to have a double mutation at codons 670/671, at the  $\beta$ -secretase cleavage site (Mullan *et al.*, 1992). The location of this mutation led to the suggestion that it acted to enhance  $\beta$ -secretase cleavages, leading to the production of more total A $\beta$ . Consistent with this hypothesis, cultured cells which express APP with the Swedish mutation (APP Swe) secrete six to eight fold higher levels of A $\beta$  peptides than cells overexpressing the wild type construct (Citron *et al.*, 1992). Furthermore, significantly increased plasma levels of A $\beta$  have been observed in some carriers of the Swedish mutation, even presymptomatically (Scheuner *et al.*, 1996). The recent identification of the  $\beta$ -secretase enzyme formally confirmed the predicted pathogenic mechanism of the Swedish mutation by demonstrating that APP Swe was a better substrate for the  $\beta$ -secretase than was APP WT (Yan *et al.*, 1999; Vassar *et al.*, 1999; Hussain *et al.*, 1999; Sinha *et al.*, 1999). The mutations in APP which have been identified to date are shown in figure 1.2.1.2.



**Figure 1.2.1.2 The mutations in APP which cause FAD or related dementias**

The sequence of the transmembrane region of APP is shown, with the Aβ sequence underlined. The mutations which have so far been found to cause FAD or related dementias are indicated in red. The double mutation at codons 670/671 (Swe) and the cluster of single mutations at codons 716 or 717 cause FAD. The mutation at codon 693 causes autosomal dominant hereditary cerebral haemorrhage with amyloid. The mutation at codon 692 causes presenile dementia and cerebral haemorrhages. Figure adapted from Selkoe, 1999.

The clinical, neuropathological and genetic aspects of FAD patients with mutations in APP have several common features. The age of onset is always before 60 years of age, inheritance is autosomal dominant and fully penetrant. Furthermore, although APP linked FAD only accounts for less than 1% of all AD cases, the clinical and pathological phenotype is indistinguishable from patients with sporadic AD. This has led to the belief that an appreciation of the pathogenic mechanisms underlying these rare APP linked FAD cases could greatly assist in the understanding of the much more common sporadic disease.

The mechanism by which the mutations in APP precipitate AD pathology has been a matter of controversy for many years. The “amyloid cascade hypothesis” predicts that the deposition of A $\beta$ 42 in the brain is neurotoxic and responsible for triggering all the other observed aspects of the neuronal degeneration (Hardy and Allsop, 1991). This hypothesis is supported by the observation that all the known pathogenic mutations in APP or the presenilins (discussed below) lead to an increase in A $\beta$ 42. Furthermore, A $\beta$  species have been reported to be neurotoxic *in vitro* and *in vivo* although there appears to be a lack of consensus regarding the mechanism by which this toxicity is mediated. It has been reported that A $\beta$  increases the concentration of extracellular calcium possibly by forming calcium channels or increasing membrane permeability (Mattson *et al.*, 1993; Arispe *et al.*, 1993; Fukuyama *et al.*, 1994). A $\beta$  has also been reported to generate reactive oxygen species (Behl *et al.*, 1994). A few years ago, it was popular to implicate reactive oxygen species in many diseases, but the observation that antioxidants cannot reproducibly attenuate A $\beta$  neurotoxicity has brought into question their relevance in this case (Lockhart *et al.*, 1994). It has been suggested that the amyloid fibrils may interact with cell surface receptors that recognise  $\beta$  sheet structures in their endogenous ligands, resulting in the aberrant activation of signal transduction pathways (Yankner, 1996). This hypothesis is particularly attractive because it could explain the observed increase in the phosphorylation of the microtubule associated protein, tau. A $\beta$  has also been reported to mediate its neurotoxicity through the activation of microglia (Meda *et al.*, 1995), the inhibition of ubiquitin-dependent protein degradation (Gregori *et al.*, 1995) and the activation (Arias *et al.*, 1995) or inhibition (Kar *et al.*, 1996) of neurotransmitter release. It is also a matter of

controversy whether A $\beta$  mediates cell death through apoptotic (Cotman and Anderson, 1995) or necrotic pathways (Behl *et al.*, 1994).

Sceptics of the amyloid cascade hypothesis cite these mechanistic discrepancies as evidence against a central neurotoxic role of A $\beta$  (Neve and Robakis, 1998). Other arguments against the hypothesis include the fact that there is poor correlation between the concentration of amyloid deposits in the AD brain and the degree of dementia. Furthermore, transgenic mouse models have shown that neurodegeneration often precedes amyloid deposition by several months and can even occur in the complete absence of amyloid (Chui *et al.*, 1999). The amyloid hypothesis also does not account for the neuroanatomical differences in plaque distribution which are characteristic of AD. An alternative hypothesis is that APP mutations cause cellular dysfunction by interfering with normal cellular trafficking or signalling by the molecule (Neve and Robakis, 1998). However, a consideration of the sites of the identified missense mutations in APP (see figure 1.2.1.2) reveals that these are exclusively clustered at the secretase processing sites. If APP mutations caused disease by affecting the normal functioning of the precursor protein, it might be anticipated that these mutations would be more randomly distributed throughout the molecule.

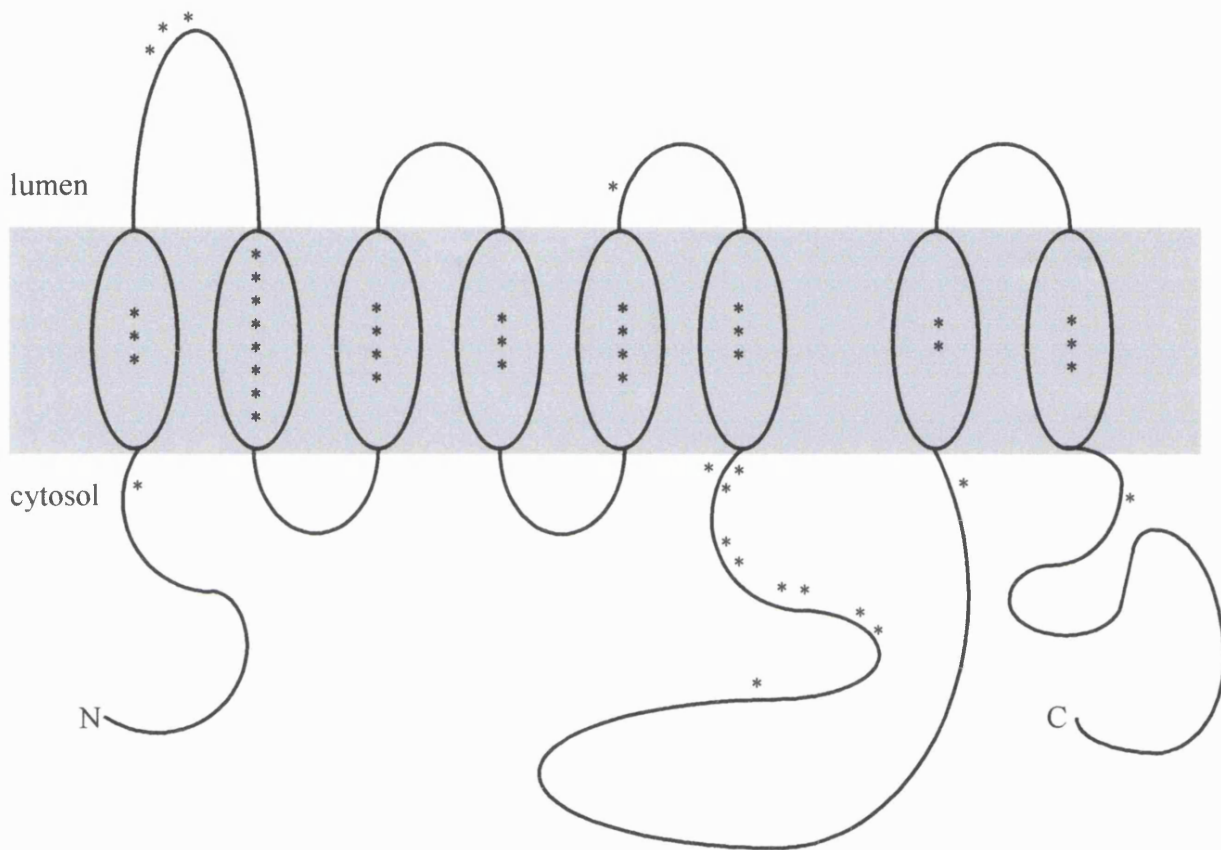
### **1.2.2 Presenilins 1 and 2 (PS1 and PS2)**

The majority of early-onset FAD pedigrees are linked to mutations in the PS1 or PS2 genes located on chromosomes 14 and 1 respectively (Alzheimer's Disease Collaborative Group, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). Mutations in the presenilin genes account for between 5 and 8% of all AD cases. These genes encode integral membrane proteins which have a sequence identity of 63%, increasing to 95% in the transmembrane (TM) domains (Lee *et al.*, 1996). A total of 49 different FAD-linked mutations have been identified in PS1 and 4 FAD-linked mutations have been found in PS2. Mutations within PS1 are responsible for the most aggressive form of FAD recorded and can cause AD pathology as early as 28 years of age. These mutations are clustered within the TM domains or the large hydrophilic loop and occur at positions conserved between the human presenilin proteins, suggesting that they affect structurally or functionally important amino acids. All the mutations identified to date



are missense mutations (one of which results in the mutation of a splice donor site and therefore results in the deletion of an entire exon,  $\Delta$ Exon9). It is interesting to note that there are no reported mutations which cause a frameshift or premature termination of the protein, suggesting that any such structural change would be lethal.

The most commonly proposed structure for the presenilins depicts eight TM domains although this is controversial. An elegant series of experiments by Li and Greenwald has lent considerable support to the eight TM structure (Li and Greenwald, 1996; Li and Greenwald, 1998). Taking advantage of the fact that  $\beta$ -galactosidase is only active in the cytosol, these authors generated a number of PS1 fusion peptides which contained this reporter gene after each putative TM domain. Using this system, it was possible to demonstrate whether each of the predicted TM domains did indeed cross the membrane. The authors concluded that the most likely structure for PS1 contained eight transmembrane domains with a further two hydrophobic regions associated with but not crossing the membrane. Although the results of this study are convincing, studies of endogenous presenilins are required to definitively confirm the predicted structure as it is possible that transient overexpression may cause abnormal membrane insertion of the protein. The proposed eight TM structure for PS1 is shown in figure 1.1.2.1 and the sites of the mutations associated with FAD are indicated by asterisks.



**Figure 1.2.2.1 Proposed transmembrane structure of PS1**

The predicted protein topology of PS1 is shown. The approximate locations of the FAD linked mutations are indicated as asterisks. The marked homology between PS1 and PS2 suggests that PS2 will have a similar structure. Figure adapted from Thinakaran, 1999.

Presenilin expression is not limited to the brain and transcripts have been detected in most peripheral tissues (Lee *et al.*, 1996). Interestingly, in the central nervous system (CNS), the highest levels of presenilin expression are observed in the hippocampus, amygdala and cerebral cortex, the areas which are most vulnerable to AD. However, expression is not limited to these areas and has been detected in brain regions which are known to remain relatively unaffected in AD. At the subcellular level, the presenilin proteins have been localised to the endoplasmic reticulum and up to the *cis* compartment of the Golgi apparatus but not beyond (Doan *et al.*, 1996; Kovacs *et al.*, 1996). Understanding the precise subcellular localisation of the presenilins has recently become one of the most critical aspects of the biology of these highly studied proteins. This point will be discussed further below.

PS1 is rapidly and efficiently proteolytically cleaved in the cytoplasmic loop domain to produce a 28kDa N-terminal fragment (NTF) and an 18kDa C-terminal fragment (CTF) which are the major species observed both *in vivo* and in cell culture (Thinakaran *et al.*, 1996). PS2 also undergoes proteolytic processing, generating a 34kDa NTF and 20kDa CTF (De Strooper *et al.*, 1997). Presenilin processing is tightly regulated and the observation that overexpression leads to the accumulation of full length presenilins but not the N and C terminal fragments has led to the suggestion that the processing may be a rate limiting step in presenilin maturation (Thinakaran *et al.*, 1996; Lee *et al.*, 1997). The large excess of overexpressed presenilin which is not proteolytically processed is targeted for degradation by the proteasome. These observations, coupled with the fact that endoproteolytic cleavage of the presenilins is conserved between species is suggestive of the fact that the processed fragments and not the full length proteins are the functional molecules. Consistent with this hypothesis, the N and C-terminal fragments accumulate in a 1:1 stoichiometry and associate into a stable, high molecular weight complex (Capell *et al.*, 1998). Importantly, however, a FAD associated PS1 mutant lacking the proteolytic cleavage site ( $\Delta$ Exon9) is still functional, both physiologically and pathogenically, despite remaining in the full length form (Steiner *et al.*, 1999). There is some tentative evidence to suggest that this  $\Delta$ exon9 variant may mimic the processed fragments, providing a possible explanation for this

apparent discrepancy (Thinakaran, 1999). In cells undergoing apoptosis, an alternative pathway for PS1 processing is mediated by the caspase family of proteases (Kim *et al.*, 1997). This cleavage occurs within the cytoplasmic loop domain but the significance of this observation is not yet known.

The first clue to the probable physiological function of the presenilins came from the observation that the *Caenorhabditis elegans* gene *sel-12* was a presenilin homologue. *Sel-12* was known to interact with LIN-12 and GLP-1, the *C. elegans* homologues of the *Drosophila melanogaster* protein, Notch (Levitan and Greenwald, 1995). Notch is a single-pass transmembrane receptor which interacts with ligands at the cell surface and then potentiates interactions with transcription factors downstream in a developmentally important and much-studied signalling pathway (reviewed in Artavanis-Tsakonas *et al.*, 1995). Mutant *sel-12* alleles cause an egg laying defect in *C. elegans* and this defect can be completely rescued by human wild type PS1 and PS2 and partially rescued by human FAD-linked PS1 variants (Levitan *et al.*, 1996). This indicated that PS1 and *sel-12* are true homologues as the wild type proteins can functionally substitute for one another across species.

Generation of PS1 knockout mice further suggested that the presenilins were involved in Notch signalling (Shen *et al.*, 1997; Wong *et al.*, 1997). Homozygous disruption of the PS1 gene (PS1<sup>-/-</sup>) was lethal at birth whereas heterozygous mutant mice showed no signs of phenotypic abnormality. The most striking phenotype of the PS1<sup>-/-</sup> mice was the severe impairment in the development of the axial skeleton. The authors observed that these skeletal abnormalities were due to an impairment of the segmentation of the somites, a defect which was detectable as early as embryonic day 9 (E9). The generation of Notch deficient mice had been reported several years previously (Conlon *et al.*, 1995) and the marked similarity between the phenotype of these animals and the PS1 knockouts was immediately noted. The Notch deficient mice had been reported to show severe skeletal abnormalities and an abnormal pattern formation of the somites. Furthermore, mice which were deficient in the vertebrate homologue of the *D. melanogaster* Notch ligand, Delta, also had a very similar phenotype (Hrabe de Angelis *et al.*, 1997). It was therefore proposed that the embryonic lethality of the PS1<sup>-/-</sup> mice was due to a defect in Notch signalling, leading to severe developmental abnormalities.

Surprisingly, mice homozygous for a null mutation in PS2 showed no phenotypic defects. However, the observation that ablation of one or both PS2 alleles on a PS1 null background was embryonic lethal at E9.5 suggested that the reason for the lack of phenotype of the PS2 knockouts was due to functional redundancy between the two presenilins (Donoviel *et al.*, 1999).

There is now a large amount of data implicating the presenilins in the Notch signalling pathway. Null mutations of the *D. melanogaster* presenilin homologue, DPS, lead to a lethal phenotype which was shown to result from a severe impairment of ligand-dependent Notch signal transduction (Struhl and Greenwald, 1999; Ye *et al.*, 1999). Furthermore, evidence for a physical interaction between Notch and DPS was recently demonstrated by the co-immunoprecipitation of the two endogenous proteins from cultured *D. melanogaster* cells (Ray *et al.*, 1999b). The Notch signalling pathway involves cleavage at an intramembranous site to release the Notch intracellular domain (NICD). NICD is then translocated to the nucleus where it forms an active transcriptional complex which activates Notch target genes. It is therefore interesting to note that this cleavage is significantly impaired in neurons and fibroblasts cultured from PS1 deficient embryos, suggesting that one of the physiological functions of PS1 is to either directly or indirectly mediate the proteolytic processing of Notch to release the functional NICD (De Strooper *et al.*, 1999). This would therefore explain why the Notch deficient, Notch ligand deficient and presenilin deficient phenotypes are so similar.

The relevance of the presenilin/Notch interaction to AD becomes apparent when a comparison between Notch and APP is made. Both proteins are single pass transmembrane proteins but more importantly, they both undergo proteolytic cleavage within the transmembrane domain, a highly unusual environment for a classical protease to function. Extending the parallels between Notch and APP, De Strooper and colleagues demonstrated that  $\gamma$ -secretase cleavage of APP is dependent on PS1. These authors used pulse-chase experiments to analyse the processing of APP in neurons cultured from PS1<sup>-/-</sup> mice and observed that the lack of PS1 caused an 80% reduction in A $\beta$  and p3 production and an accumulation of  $\alpha$  and  $\beta$ -stubs (De-Strooper *et al.*, 1998). Referring back to the pathway of APP processing shown in figure 1.2.1.1, it can be seen

that such an outcome is clearly indicative of a deficiency in  $\gamma$ -secretase activity. APLP1 has also been shown to undergo a PS1-dependent intramembranous cleavage event (Naruse *et al.*, 1998). The simplest explanation of these observations is that PS1 is itself the elusive  $\gamma$ -secretase and is responsible for the intramembranous cleavages of Notch, APLP1 and APP. This was the original explanation proposed by De Strooper *et al.* and has been a much debated issue ever since.

The most convincing evidence in favour of De Strooper's provocative model was recently provided by the demonstration that two aspartate residues in TM domains 6 and 7 of PS1 and PS2 are critically required for  $\gamma$ -secretase activity (Wolfe *et al.*, 1999; Kimberly *et al.*, 2000). These authors reported that mutating either of these two transmembrane aspartate residues either to alanine or even to glutamate (a conservative substitution) led to a significant reduction in A $\beta$  and p3 production and a corresponding increase of the  $\alpha$  and  $\beta$ -stubs. It was also observed that mutation of these ~~alanine~~<sup>aspartate</sup> residues prevented the normal proteolytic processing of the presenilins to the N and C-terminal fragments, leading the authors to suggest that the proteins might be autoactivated intramembraneous aspartyl proteases. Furthermore, microsomes prepared from cells expressing wild type PS1 but not PS1 with the mutated aspartate residues could support  $\gamma$ -secretase site cleavage of  $\alpha$  and  $\beta$  stubs. Although this experiment would tend to suggest that PS1 is the  $\gamma$ -secretase or is an essential co-factor for  $\gamma$ -secretase, there are several problems with this attractive hypothesis. The most pressing problem is a practical one. PS1 is known to be exclusively located in the ER and cis-Golgi (Doan *et al.*, 1996; Kovacs *et al.*, 1996; Annaert *et al.*, 1999) whereas the production of the major A $\beta$  species (and thus the site of  $\gamma$ -secretase activity) is in the late secretory compartments and endocytotic pathway (Hartmann *et al.*, 1997). Likewise, the intermembranous cleavage of Notch is thought to occur near the cell surface (Schroeter *et al.*, 1998). It has been argued that this spatial paradox is not an issue, with models proposed suggesting a subcellular transport pathway to recycle the  $\alpha$  and  $\beta$ -stubs back to the ER for PS1- $\gamma$ -secretase cleavage (Annaert and De Strooper, 1999). It has also been suggested that PS1 may bind to immature APP or Notch in the

ER and that the resulting complex is then trafficked to the cell surface where PS1 effects the cleavage (Ray *et al.*, 1999a).

Alternative hypotheses include the suggestion that PS1 influences the trafficking of APP and Notch such that they are co-compartmentalised with an unidentified  $\gamma$ -secretase (Thinakaran, 1999). This theory is supported by default in a recent publication which used baculovirus to overexpress APP and PS1 in insect cells in which endogenous  $\gamma$ -secretase activity is not detected (Octave *et al.*, 2000). PS1 was proteolytically cleaved and the co-immunoprecipitation of APP and PS1 demonstrated that the two proteins were able to interact in these cells. Western blotting showed that  $\alpha$  and  $\beta$ -stubs were generated but that these were not further processed to p3 or A $\beta$ , demonstrating that PS1 might be necessary but is not sufficient for  $\gamma$ -secretase activity in these cells. The authors concluded that PS1 was not the  $\gamma$ -secretase and favoured a model where PS1 is involved in the trafficking of APP. Although interesting, this paper does not preclude the possibility that the insect cells are lacking an additional factor or factors required for the secretase activity of PS1 or do not make a necessary post-translational modification of PS1.

Alternatively, it has been suggested that PS1 somehow “marks” immature APP and Notch in the ER for cleavage by a  $\gamma$ -secretase later in the secretory pathway (Annaert and De Strooper, 1999). A recent meeting report with the subheading “ $\gamma$ -secretase: we all had it in the freezer” indicates that some laboratories are already convinced about the nature of the elusive enzyme (Haass and Mandelkow, 1999). However, formal proof or otherwise of presenilins as the  $\gamma$ -secretase awaits the reconstitution of a pure presenilin and its substrate into a truly cell free system such as liposomes.

The mechanisms by which mutations in PS1 and PS2 cause FAD are not known. However, it is apparent that the mutations are toxic “gain of function” rather than “loss of function” mutations. This conclusion is based on the absence of nonsense or frameshift mutations in both PS1 and PS2. Furthermore, an FAD-linked PS1 allele was found to completely rescue the severe developmental abnormalities of the PS1 knockout mouse (Davis *et al.*, 1998; Qian *et al.*, 1998). It is now widely accepted that this toxic gain of function leads to an increase in the production of the longer, more

amyloidogenic form of A $\beta$ , A $\beta$ 42. The earliest evidence for this was the observation that the levels of A $\beta$ 42 but not total A $\beta$  were significantly elevated in the plasma and conditioned media from fibroblasts of individuals carrying PS1 mutations (Scheuner *et al.*, 1996). This result was confirmed by analysis of the media of cells transfected with cDNAs encoding mutant PS1 and analysis of the A $\beta$  in the brains of transgenic mice overexpressing mutant PS1 (Duff *et al.*, 1996; Borchelt *et al.*, 1996). This data is clearly entirely consistent with the now known correlation between presenilins and  $\gamma$ -secretase; an aberrant gain of function mutation in the presenilins either directly or indirectly leads to enhanced  $\gamma$ -secretase activity, resulting in increased production of A $\beta$ 42. However, the mechanism by which this gain of toxic function is mediated is unknown.

It has been reported that PS1 forms a complex with and stabilises  $\beta$ -catenin (Zhang *et al.*, 1998b). Pathogenic mutations in PS1 are less able to carry out this stabilising interaction and increased degradation of  $\beta$ -catenin has been observed in the brains of transgenic mice and human patients with AD. It was hypothesised that mutations in the PS1 gene may predispose individuals to AD by reducing the stability of  $\beta$ -catenin and potentiating neuronal apoptosis. In contrast, a more recent study demonstrated that the half-life of  $\beta$ -catenin was significantly longer in PS1<sup>-/-</sup> cells and that expression of wild-type but not FAD-associated mutant PS1 increased the turnover of  $\beta$ -catenin (Kang *et al.*, 1999). The reason for the major discrepancies between these two reports is unclear. It has also been reported that PS1 can affect the nuclear translocation of  $\beta$ -catenin (Nishimura *et al.*, 1999). The fact that both  $\beta$ -catenin and a tau phosphorylating enzyme, glycogen synthase kinase (GSK) are involved in the same signalling pathway (Wnt signalling) suggests that this issue may be an important link between the two AD pathologies and will therefore have to be resolved.

There are numerous reports proposing a role for PS1 in apoptosis but these reports are divided as to whether the protein has a pro-apoptotic (Guo *et al.*, 1997; Wolozin *et al.*, 1998; Czech *et al.*, 1998) or anti-apoptotic (Bursztajn *et al.*, 1998; Roperch *et al.*, 1998) effect. The data implicating PS2 in apoptosis are more consistent. Overexpression of PS2 in NGF-differentiated PC12 cells significantly increases apoptosis initiated by trophic factor withdrawal (Wolozin *et al.*, 1996; Janicki and



Monteiro, 1997). This effect is abrogated by the overexpression of a PS2 antisense cDNA and enhanced by the overexpression of a FAD-associated PS2 mutation. The recent finding that presenilins interact with the anti-apoptotic protein Bcl-xL hints at a potential mechanism by which the presenilins might regulate apoptosis (Passer *et al.*, 1999).

### 1.2.3 Apolipoprotein E (ApoE)

The first realisation that genetic factors could predispose an individual to AD was made in 1993 with the observation that allelic variation in the ApoE gene correlated with the susceptibility of an individual to late onset AD (Strittmatter *et al.*, 1993a). ApoE is a 34kDa very low density lipoprotein synthesised systemically by the liver and in the CNS by astrocytes and microglia (Weisgraber and Mahley, 1996). ApoE is thought to play a role in the redistribution of lipid and cholesterol during membrane repair and has been proposed to be important in maintaining synaptic plasticity, especially after neuronal injury (Boyles *et al.*, 1989; Poirier *et al.*, 1993). ApoE is located on chromosome 19 and has three major alleles, E2, E3 and E4, with ApoE3 being the most prevalent. Patients with late onset AD were found to have a disproportionate increase in the frequency of the ApoE4 allele (Strittmatter *et al.*, 1993a). Unlike the mutations in APP and PS1 which, although causative, only account for a total of 10% of all AD cases, ApoE4 has been reported to increase the age-adjusted relative risk of developing AD by 3 to 10 fold in heterozygotes and homozygotes respectively (Weisgraber and Mahley, 1996). The ApoE2 allele has been reported to be associated with reduced risk of developing AD and may therefore act as a protective factor (Corder *et al.*, 1994).

A number of different hypotheses have been proposed to explain how the various ApoE isoforms might affect the development of AD. These include isoform specific neurotoxic (Tolar *et al.*, 1999) or neurotrophic (Holtzman *et al.*, 1995) properties, effects on tau phosphorylation (Strittmatter *et al.*, 1994) or antioxidative properties (Miyata and Smith, 1996). However, the only consistently observed alteration in the neuropathology of AD patients with an ApoE4 allele is an increase in the number of amyloid plaques (Schmechel *et al.*, 1993). Asymptomatic ApoE4 allele carriers also display increased amyloid formation, suggesting that ApoE4 may promote A $\beta$

deposition independently of other features of AD (Polvikoski *et al.*, 1995). *In vitro*, ApoE binds to A $\beta$  with high affinity and in some studies the ApoE4 isoform bound more rapidly and promoted A $\beta$  fibrillogenesis more potently than the ApoE3 isoform (Strittmatter *et al.*, 1993b). These *in vitro* observations are supported by immunohistochemical studies which have indicated that ApoE is associated with amyloid plaques in the AD brain *in vivo* (Rebeck *et al.*, 1993). From these observations, it could be proposed that isoform specific differences in ApoE production at an incipient stage of A $\beta$  deposition may catalyse the rate of fibril formation, accelerating the rate of onset of AD.

Convincing support for this hypothesis has recently been provided by the striking phenotype of an ApoE knockout mouse overexpressing APP with an FAD-associated mutation (Bales *et al.*, 1999). This mouse showed no A $\beta$  deposition even at 22 months of age whereas a mouse with an uninterrupted ApoE locus overexpressing the same APP gene showed extensive A $\beta$  deposition at 9 months of age (V717F mouse, Games *et al.* 1995, discussed in section 1.3.1). Furthermore, A $\beta$  immunoreactivity in the hippocampus of the transgenic mice was reduced in an ApoE gene dose dependent manner. The authors demonstrated that the absence of ApoE did not affect the transcription or translation of APP or its processing to A $\beta$ . In fact, the ApoE knockout mice overexpressing the FAD-associated APP had significantly higher levels of A $\beta$  than wild type mice, suggesting that the lack of ApoE had either prevented the maturation of this A $\beta$  into fibrillar deposits or had enhanced the clearance of such deposits. The same paper also reports preliminary studies on the contribution of specific ApoE alleles to the phenotype of the APP mutant transgenic mice. A 10-fold greater amount of amyloid deposition was observed in 15 month old mice with an ApoE4 allele as compared to those with an ApoE3 allele. This interesting observation suggests that the effects of ApoE on A $\beta$  deposition are both qualitative (isoform specific) and quantitative (expression level dependent). Recently, a number of polymorphisms in the ApoE gene promoter have been identified and linked to increased risk of AD (Lambert *et al.*, 1998; Bullido *et al.*, 1998). These polymorphisms have been found to lead to increased promoter activity in transient expression assays (Artiga *et al.*, 1998). This observation,

coupled with the quantitative effects of ApoE gene dosage described by Bales and colleagues, reveals the probable mechanism by which these promoter polymorphisms predispose to AD.

With the identification of FAD pedigrees with mutant APP, PS1 or PS2 genes and the realisation that these mutations were 100% penetrant, the late 1990's saw research attention shift away from ApoE. However, the identification of this important link between APP, A $\beta$  and ApoE should help to redress this balance.

#### 1.2.4 $\alpha$ 2-macroglobulin ( $\alpha$ 2M)

$\alpha$ 2M is a serum protease inhibitor which acts by sterically trapping the target enzyme. The gene for  $\alpha$ 2M is on chromosome 12 and polymorphisms in this gene have been identified as genetic risk factors for AD (Blacker *et al.*, 1998). Biological support of the proposed  $\alpha$ 2M and AD association comes from the observation that functional  $\alpha$ 2M is able to prevent A $\beta$  fibril formation *in vitro* (Hughes *et al.*, 1998). Furthermore, AD patients who are carriers of the  $\alpha$ 2M polymorphisms have significantly more A $\beta$  in their brains than non-carriers (Tanzi, 1999).

### 1.3 Modelling AD

For many years, a major obstacle to elucidating any of the pathogenic mechanisms involved in AD was the lack of an animal model. The identification of FAD-associated mutations in APP and PS1 led to the generation of a number of transgenic mice harbouring mutations in one or both of these genes. Although these mice have greatly assisted in the understanding of AD, no model produced so far comprehensively reflects the pathological and behavioural features of the human disease.

The mouse models which have been generated to date can be broadly divided into four categories; those which overexpress full length mutant APP, those which overexpress only A $\beta$  or the C-terminal 100 amino acids of APP (the  $\beta$ -stub), those which have FAD associated mutations in PS1 and finally those generated by crosses between the first and third of these categories, namely mice simultaneously overexpressing wild type or mutant human APP and PS1. Representative examples from each of these categories are discussed below.

### 1.3.1 Overexpression of full length mutant APP

The first transgenic mouse to produce any of the pathological features of AD was produced by Athena Neurosciences in 1995 (Games *et al.*, 1995). This group used a platelet derived growth factor (PDGF) promoter to drive the expression of a human APP minigene harbouring the FAD-associated V717F mutation described in section 1.2.1. This system allowed all three isoforms of APP to be produced. The resulting transgenic animals had a neuropathological phenotype closely resembling AD, with both diffuse A $\beta$  deposits and mature plaques, dystrophic neurites and synapse loss in frontal and hippocampal areas. As would be expected for the APP 717 mutant allele, A $\beta$ 42 was the major amyloid species in these mice. However, no behavioural deficits were observed and no neurofibrillary tangles or hyperphosphorylated tau isoforms were detected.

APP transgenic mice which exhibited behavioural deficits were first reported in 1996 (Hsiao *et al.*, 1996). These mice were generated by using the hamster prion protein (PrP) promoter to overexpress human APP containing the Swedish mutation. The levels of A $\beta$ 40 and A $\beta$ 42 were reported to be increased 5- and 14-fold respectively when compared to non-transgenic control animals. Neuritic A $\beta$  deposits were detected in the amygdala, hippocampus and cortex of these mice by 12 months of age but no tau neuropathology was observed. Later studies on this transgenic line have suggested that oxidative stress may be an important mediator of some aspects of the neurotoxicity observed (Smith *et al.*, 1998a). The advantage of this transgenic model over the one reported by Games *et al.* is that the mice were reported to show impairments in several learning and memory tests. Importantly, these behavioural deficits were apparent by 9-10 months of age, several months before the appearance of amyloid plaques. It should be noted that some of the interpretations of the behavioural data on these mice have been challenged (Routtenberg, 1997).

The Thy1 promoter has also been used to drive the expression of a human APP transgene containing either the Swedish mutation or the Swedish mutation plus the V717I mutation (Sturchler-Pierrat *et al.*, 1997). In addition to showing neocortical A $\beta$  deposition, these mice demonstrated several further interesting features. Firstly, distorted acetylcholinesterase fibres were detected associated with dystrophic neurites

surrounding the core of A $\beta$  deposits. Cholinergic neurons are known to be severely compromised in AD and this is the first time that any aspect of this pathology has been reproduced in a transgenic model. Secondly, dystrophic neurites surrounding the A $\beta$  deposits were found to contain hyperphosphorylated tau species. Although the effect was subtle and no neurofibrillary tangles were observed, this important observation provided the first *in vivo* link between the two pathologies of AD. Further work on these mice revealed age-dependent deposition of A $\beta$  in the cerebral vasculature, another feature of human AD which had not been previously reproduced in a mouse model (Calhoun *et al.*, 1999). Unfortunately, no behavioural abnormalities were observed in these mice.

Mice overexpressing human APP isoforms have also been generated by an alternative transgenesis technique. Lamb and colleagues generated a yeast artificial chromosome (YAC) containing the genomic APP sequence with the Swedish or V717I mutation or both and used this to establish transgenic lines (Lamb *et al.*, 1997). This model has the advantage that it expresses all the alternatively spliced isoforms of APP with the correct expression pattern. This transgenic line showed increased levels of all A $\beta$  peptides in the brain. When the APP contained the V717I mutation the levels of A $\beta$ 42 were specifically elevated, consistent with enhanced  $\gamma$ -secretase cleavage.

Recently, the APP transgenic mouse described by Games *et al.* was used to study the effects that immunisation with A $\beta$ 42 had on disease progression in these mice (Schenk *et al.*, 1999). Transgenic animals were immunised either before the onset of neuropathology (at 6 weeks) or when A $\beta$  deposition and subsequent neuropathologic changes were established (11 months). It was demonstrated that immunisation of the young animals prevented the development of A $\beta$  plaque formation, neuritic dystrophy and gliosis. Furthermore, treatment of the older animals significantly reduced the extent and progression of these pathologies. Animals which began treatment at 11 months of age showed greater than 99% reduction of A $\beta$ 42 burden at 18 months of age compared with unimmunised littermates. The authors demonstrated that the A $\beta$  production itself was unaffected, suggesting that the immunisation either prevented the deposition of or enhanced the clearance of the A $\beta$  peptides. Although this was a remarkable observation,

the conclusion by the authors that A $\beta$  immunisation may prove beneficial for both the treatment and prevention of human AD is at best premature, not least because it is currently unknown whether A $\beta$  causes AD.

### **1.3.2 Overexpression of A $\beta$ or the C-terminus of APP ( $\beta$ -stub)**

Overproduction of rat A $\beta$ 42 under the control of the strong, neurofilament light chain promoter generated mice which showed widespread immunoreactivity for A $\beta$ , apoptosis and reactive gliosis and had a 50% reduced lifespan compared to non-transgenic controls (LaFerla *et al.*, 1995). The similarity of this phenotype to that of the APP Swe overexpressing mice (Hsiao *et al.*, 1996) led to the suggestion that A $\beta$ 42 overexpression was directly responsible for the pathology observed in these APP mutant mice. Although this conclusion might be valid, it is important to note that both these transgenic mice strains were bred onto the same genetic background (FVB/N) and that genetic background has previously been shown to be an important modulator of phenotype in AD models (Carlson *et al.*, 1997). Furthermore, the model published by LaFerla and colleagues has come under some criticism due to the fact that the A $\beta$  peptide was not secreted and therefore does not accurately represent the situation occurring in AD.

Several groups have generated mice overexpressing the C-terminal 100 or 104 amino acids of human APP (the  $\beta$ -stub or the  $\beta$ -stub plus four amino acids). When the C-terminal 104 amino acids of APP were overproduced under the control of the neurofilament light chain regulatory sequences, the mice developed increased gliosis and microglial reactivity as well as a 20% cell loss in the CA1 region of the hippocampus (Nalbantoglu *et al.*, 1997). An age dependent decline in spatial learning and long term potentiation (LTP) was also reported. However, no A $\beta$  production could be detected in these mice. This observation is probably explainable by the fact that these authors did not include a signal peptide on their transgene, thereby preventing it from entering membrane compartments of the cell where it could interact with the secretases. This paper therefore raises the important issue of neurodegeneration in the absence of A $\beta$ , suggesting that in this case at least the APP related neuronal dysfunction may have

arisen by A $\beta$  independent mechanisms. In contrast, when transgenic mice were generated from a  $\beta$ -stub transgene bearing a signal peptide, A $\beta$  overproduction was observed (Sberna *et al.*, 1998). However, the animals showed no significant amyloid deposition and an increase in acetylcholinesterase activity was detected, the opposite effect to that seen in AD.

### 1.3.3 Overexpression of mutant PS1 or PS2

Within 16 months of the realisation that mutations in PS1 were linked to FAD, the phenotypes of transgenic mice containing these mutations were published. The first such model used the PDGF promoter to overexpress human wild type PS1 or PS1 with the mutations M146L or M146V (Duff *et al.*, 1996). Analysis of these mice demonstrated that mutant, but not wild type, PS1 led to an over-representation of A $\beta$ 42 without affecting levels of total A $\beta$ . The effect was subtle, with the difference in A $\beta$ 42 load between PS1 mutant and PS1 wild type overexpressing animals being approximately 1.6 fold. A number of other transgenic lines which overexpress mutant PS1 have also been established (Borchelt *et al.*, 1996; Citron *et al.*, 1997; Qian *et al.*, 1998). Despite using different promoters to drive PS1 expression, different FAD-associated mutations and different strains of mice, all these studies demonstrated a clear increase in the ratio of A $\beta$ 42 to A $\beta$ 40. FAD-associated PS2 transgenic mice have also been generated and these mice also demonstrated a specific increase in A $\beta$ 42 with no change in total A $\beta$ .

The brains of mice overexpressing mutant PS1 or PS2 do not show any A $\beta$  deposition, even at 17 months of age (Chui *et al.*, 1999). There are several potential explanations for this observation. Firstly, because APP is not being overexpressed, it is possible that the total amount of A $\beta$  produced in these mice may be below a certain threshold level required for amyloid deposition. Alternatively, the lack of plaques could be attributable to subtle differences between rodent and human A $\beta$ . Convincing support for the latter hypothesis comes from the observation that rodent A $\beta$  is less amyloidogenic than human A $\beta$  *in vitro* (De-Strooper *et al.*, 1995) and that transgenic mice overexpressing murine A $\beta$  show some diffuse amyloid deposits but no plaques

(LaFerla *et al.*, 1995). Interestingly, despite the lack of A $\beta$  deposition, the PS1 transgenic mice show significant age-dependent neuronal loss (Chui *et al.*, 1999). This observation led these authors to conclude that downstream events in the amyloid cascade, such as neuritic plaques and inflammatory responses are not prerequisites for the induction of neuronal degeneration.

#### **1.3.4 Simultaneous overexpression of mutant APP and mutant PS1**

The mice described in section 1.3.3 examined interactions between mutant human PS1 and murine APP. In order to explore the effect of mutant alleles of PS1 on the processing of human APP in transgenic mice, several groups established double transgenic lines (Borchelt *et al.*, 1996; Citron *et al.*, 1997). The hippocampi of mice which contained a mutant human PS1 allele (M146L or L286V) and wild type human APP were found to have a significantly higher ratio of A $\beta$ 42 to A $\beta$ 40 than control mice overexpressing WT alleles of both genes. These reports confirmed the hypothesis that mutant PS1 affects the processing of APP.

In order to examine the effects of simultaneous FAD-linked mutations in APP and PS1, mice which contained mutated copies of both human genes were generated (Holcomb *et al.*, 1998). These doubly transgenic mice performed worse in some of the spatial memory tests than did either of the singly transgenic control groups. It was reported that the ratio of A $\beta$ 42 to A $\beta$ 40 was significantly greater in mice containing APP Swe and either PS1 M146L or A246E compared to mice overexpressing APP Swe only or APP Swe and wild type PS1. This effect was detectable as early as 3 months of age. YAC transgenic technology has also been employed to produce mice simultaneously overexpressing all isoforms of mutated human APP and mutated human PS1 (Lamb *et al.*, 1999). These animals also showed significantly increased levels of A $\beta$ 42 and robust amyloid deposition but no behavioural data was reported.



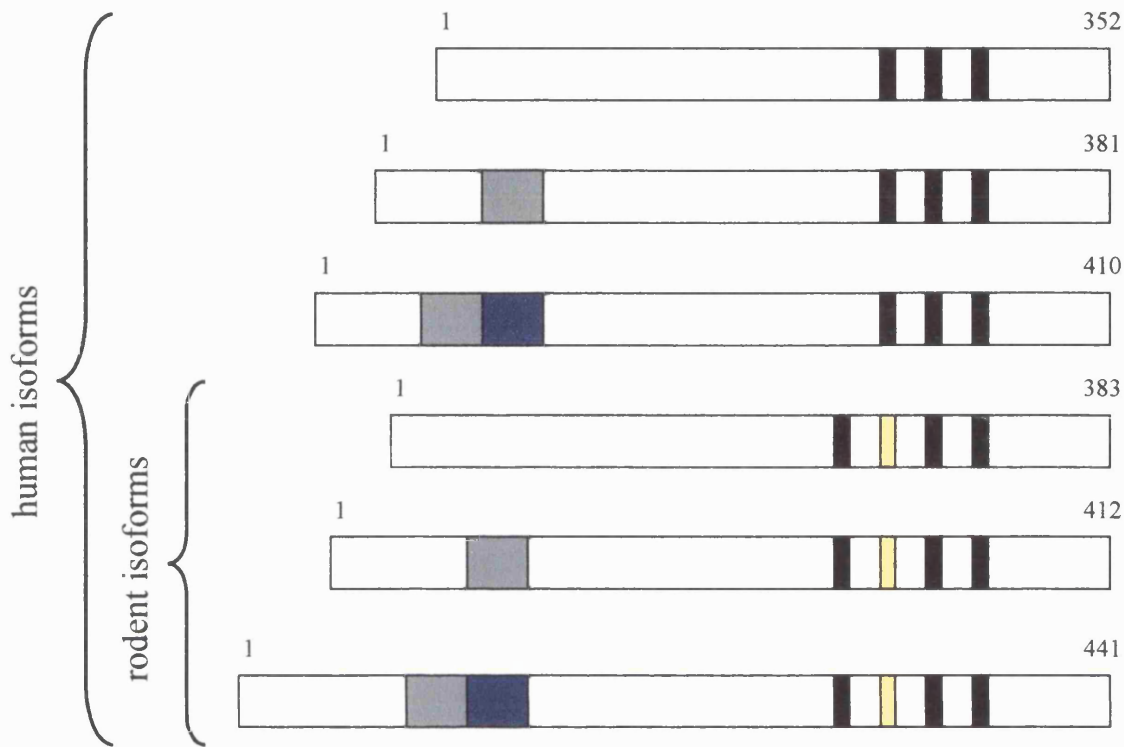
### 1.3.5 The scope and limitations of rodent models of AD

The plethora of transgenic mice available is symptomatic of the complexity and lack of understanding of AD. For a mouse model to be valuable it should demonstrate an age-dependent decline in complex cognitive behaviour and show important pathological features of the disease. None of the mouse models discussed completely reproduces the histopathological, biochemical and cognitive impairments characteristic of AD. The major omission in all the current models is the neurofibrillary tangles of the microtubule associated protein, tau. One of the APP transgenic mouse models demonstrated subtle changes in tau phosphorylation (Sturchler-Pierrat *et al.*, 1997) and excitotoxic brain insults in rodents have been shown to induce changes in tau (Stein-Behrens *et al.*, 1994) but the stable twisted tau filaments characteristic of AD are not seen. Although the transgenic models described have been invaluable in dissecting some of the pathogenic mechanisms of AD, there is some evidence to suggest that rodents may be inherently unsuitable for modelling this multifactorial human disease.

Aged rodents do not naturally develop AD-like disorders, which is in contrast to a number of other species including polar bears, primates, dogs and sheep (Selkoe *et al.*, 1987; Nelson *et al.*, 1994). Furthermore, rodent A $\beta$  does not aggregate *in vitro* and transgenic mice overexpressing murine A $\beta$  do not develop plaques (De Strooper *et al.*, 1995). More importantly, considering the major omission from all the currently available transgenic models of AD, rodents only contain a subset of the tau isoforms which have been implicated in the human disease.

Tau is expressed from a single gene on chromosome 17. In the adult human brain, six isoforms of tau ranging between 352 and 441 amino acids in length are produced as a result of alternate splicing. A schematic representation of these isoforms is shown in figure 1.3.5.1. The incorporation or exclusion of exon 2 or exons 2 and 3 results in proteins with 0 (0N), 29 (1N) or 58 (2N) amino acid inserts near the N-terminus. Furthermore, exon 10 can be alternatively spliced to give products with either three (3R) or four (4R) microtubule binding repeats. Additional alternate splicing and inclusion of exon 4A can yield a group of higher molecular weight tau, known as “big tau”. In adult human brain, 3R tau is slightly more abundant than 4R tau and there are

more 0N and 1N isoforms than 2N isoforms (reviewed in Spillantini and Goedert, 1998). Abnormally aggregated tau isolated from human AD brains contains all six tau isoforms (Goedert *et al.*, 1992). In the context of developing transgenic models of AD it is therefore of paramount importance to note that rodents only express 4R tau (Gotz *et al.*, 1995).



**Figure 1.3.5.1 Tau isoforms of the human and rodent brains**

A schematic representation of the tau isoforms present in adult human and adult rodent brain is shown. The alternatively spliced amino terminal inserts are shown in grey (exon 2) and blue (exon 3). The microtubule binding repeats are shown in black with the alternatively spliced repeat (exon 10) in yellow. All six isoforms are expressed in adult human brain whereas only the four repeat isoforms are expressed in rodent brains. The paired helical filaments seen in post mortem AD brains contain hyperphosphorylated forms of all six isoforms. Figure adapted from Spillantini and Goedert, 1998.

Following the identification of the FAD-associated mutations in APP and PS1 and the realisation that these all acted to increase A $\beta$  deposition, neurofibrillary tau tangles were often considered to be a downstream or non-specific aspect of AD pathology. However, a non-Alzheimer's dementia called fronto-temporal dementia with Parkinsonism related to chromosome 17 (FTDP-17) has recently been found to be caused by mutations in the tau gene (Hutton *et al.*, 1998). Four exonic and four intronic mutations have been described in thirteen families. The exonic mutations are located in the microtubule binding repeat region, suggesting that they may serve to reduce the ability of tau to interact with microtubules. The intronic mutations are located close to the splice site of the intron following exon 10 (shown in yellow in figure 1.3.5.1). These intronic mutations were shown to increase the ratio of tau mRNAs containing exon 10 and therefore the proportion of tau isoforms with 4 microtubule binding repeat domains (4R) (Grover *et al.*, 1999). This alteration in the ratio of 4R relative to 3R tau is sufficient to lead to the development of a filamentous tau pathology and dementia. Despite the fact that no cases of AD have been associated with mutations in tau, this important discovery has suggested that in at least some AD cases, tau dysfunction is likely to be a cause, not just a by-product, of the neuropathology.

The response of the AD field to the identification of these tau mutations is the proposal to generate transgenic mice expressing one of the mutant tau isoforms and cross this line with the double mutant APP/PS1 mice (Selkoe, 1999). Although this will almost undoubtedly produce the long-sought pathology of both amyloid plaques and hyperphosphorylated tau tangles, the relevance of a mouse model containing extremely rare mutations in three different genes is questionable. A more pertinent model might be one which utilised the YAC technology described in section 1.3.1 to express the entire genomic clone of wild type human tau on the background of a tau knockout mouse expressing wild type human APP and mutant human PS1 or vice versa. Whilst such a model might address the issue of species specific tau isoforms, it is possible that there are many other aspects to this complex multifactorial disorder which cannot be fully recreated in a species which does not naturally develop AD or related disorders. It is apparent from the foregoing discussion that it might be preferable to model AD in a

species with a longer life span and in which the aged population naturally became more susceptible to neurodegeneration.

Due to the current limits of transgenesis, there has been very little work reported on attempts to model AD in species other than rodents. However, it has been found that A $\beta$  fibrils directly injected into the cerebral cortex of old but not young rhesus monkeys can cause neurodegeneration and changes in tau phosphorylation (Geula *et al.*, 1998). Notably, similar A $\beta$  injections in the brains of young or old rats produced neither of these effects (Games *et al.*, 1992; Geula *et al.*, 1998).

A secondary limitation of transgenic technology is that the introduction of genes into the germline is a somewhat uncertain and time-consuming procedure. Importantly in the study of a complex disorder such as AD, the study of gene combinations then requires the breeding and subsequent crossing of multiple transgenic lines.

#### **1.3.6 Direct delivery of AD related genes to post-mitotic neurons**

It is apparent from the preceding section that a system which facilitated the introduction and long term expression of exogenous genes directly into post mitotic neurons would appear to offer improvements to both cell culture and animal studies of AD. Such a system would allow the rapid evaluation of novel genes or gene combinations *in vitro* or *in vivo* without the need for the generation and crossing of several transgenic lines. Furthermore, the system could provide a means for rapidly screening for the most aggressive mutation or combination of mutations. Most importantly, this approach could be used to study gene interactions relevant to AD *in vivo* in a non-rodent model.

Post-mitotic neurons are notoriously hard to transfect. Gene transfer to neurons in culture was traditionally achieved by microinjecting individual neurons. This laborious procedure has been largely superseded by the development of various vector systems designed to transduce non-dividing cells with high efficiency.

#### **1.4 Vector systems for delivering genes to neurons**

An ideal vector system for delivering genes to neurons would allow efficient gene delivery and high level, long term expression of the transgene without causing toxicity to the target cell or eliciting an immune response in the host. The fact that these criteria

have proven very difficult to meet has led to the development of a variety of different gene delivery systems. These can be broadly divided into non-viral and viral techniques. The advantages and disadvantages of each system will be discussed below.

#### **1.4.1 Non-viral vectors**

The process of transfecting naked DNA into cells is highly inefficient. Firstly, cellular uptake is non-specific, probably relying on receptor mediated endocytosis. Once inside the cell, extensive lysosomal breakdown of DNA occurs in the cytoplasm and entry into the nucleus is undirected. In the nucleus the DNA is susceptible to further degradation and stable integration into the host genome only occurs very rarely. Synthetic vectors have been designed to increase the efficiency of every stage of this process and some of these improvements are discussed below.

Cellular uptake can be improved by affixing the plasmid DNA to gold particles and then firing the complex into the tissue (Klein and Fitzpatrick-McElligott, 1993). These 'DNA projectiles' can penetrate cells without killing them, thereby bypassing the endocytotic route. Successful transfer of exogenous genes into primary neuronal cultures and *ex vivo* gene transfer into brain tissue have been reported using this technique (Jiao *et al.*, 1993).

Cationic liposomes are usually formulations of two lipids, one of which is cationic and the other a neutral helper. The liposomes form a complex with the DNA, condensing it and protecting it from degradation (Felgner *et al.*, 1987). These 'lipoplexes' can transfect a wide variety of cell types as their non-specific interaction with cell membranes is mediated only by the excess positive charge on the surface of the lipoplex. The lipoplex is then taken up by endocytosis and the DNA must then escape from the endosomal lysosomal pathway, an event which is facilitated by the helper lipid forming tubules through which the DNA can be released into the cytosol. Due to the non-specific nature of the interaction between the cationic liposome and the DNA, there is no limit on the size of the exogenous gene to be introduced. Furthermore, the host does not direct an immune response to the transfected cells and the lipoplex is relatively non-toxic.

Advances in liposome technology have included the incorporation of neutral fusogenic proteins such as influenza virus hemagglutinin subunit HA2 or Sendai virus fusion protein (Wagner *et al.*, 1992; Tomita *et al.*, 1996). The fusogenic viral liposome can then interact with cells, taking advantage of the fusion properties of the virus to transport the lipoplex into the cytoplasm. A further addition to this system is the addition of a nuclear protein to the DNA prior to being complexed with the cationic liposomes. These nuclear proteins include high mobility group protein 1 (HMG 1) or the SV40 large T antigen nuclear localisation signal (Kaneda *et al.*, 1989; Fritz *et al.*, 1996). These proteins serve to direct the DNA toward the nucleus, potentially increasing the expression of the transgene.

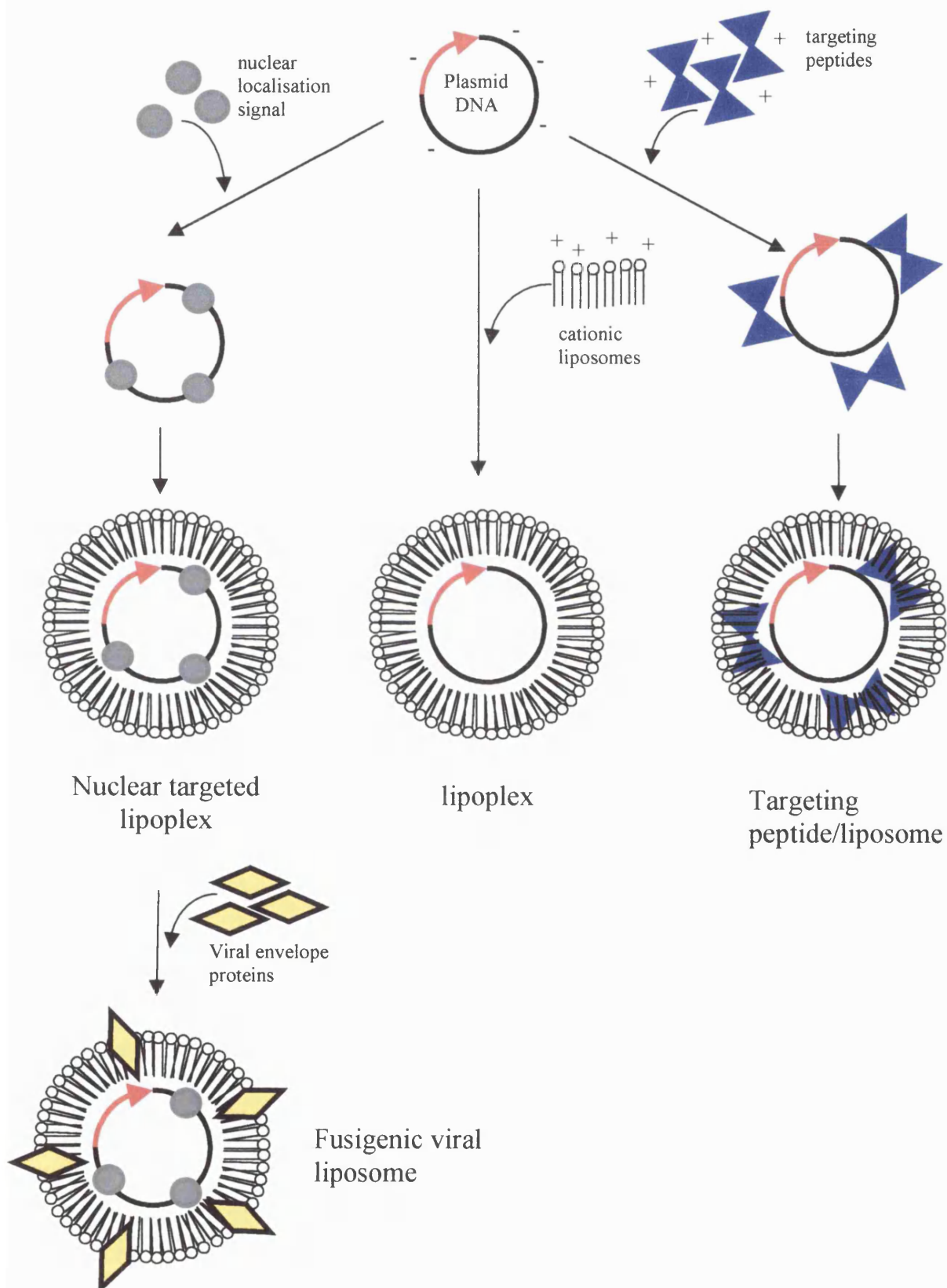
The observation that integrin mediated cell attachment and entry is a common pathway for a number of viruses led to the idea of using integrin to assist liposome mediated gene delivery. Integrin targetting peptides contain an integrin binding domain (usually an arginine-glycine-aspartic acid (RGD) motif) and a DNA-binding domain (usually polylysine) (Hart *et al.*, 1995; Harbottle *et al.*, 1998). Integrin targeting peptides can be used as a separate vector system but are often complexed with cationic liposomes to improve transfer efficiency. Entry into the cell is mediated through interactions with cell surface integrin receptors. This system has also been reported to improve the longevity of transgene expression, possibly because the polylysine domain bears some resemblance to nuclear localisation signals and the DNA-peptide complex is more resistant to degradation than DNA alone. Furthermore, as the interaction between the peptide and DNA is mediated through charge interactions, there is no limit to the size of the transgene which can be transfected. A schematic representation of these synthetic vectors is shown in figure 1.4.1.1.

Despite these improvements, the non-viral technologies described above offer only low transduction efficiencies, especially *in vivo*.

#### **Figure 1.4.1.1 Synthetic liposome-based vectors**

Plasmid DNA containing the transgene (red arrow) is complexed with cationic liposomes. Prior to complexing, the DNA can be bound to nuclear localisation peptides (such as HMG 1) or electrostatically attracted to targeting peptides (such as ligands for cell surface integrin receptors). The liposome complexes can also be bound to fusogenic viral proteins in order to mediate more efficient fusion with the cell membrane.





### 1.4.2 Viral vector systems

The low transduction efficiencies achieved by the synthetic vectors described in the preceding section have limited their use as gene delivery systems, especially *in vivo*. An increasing number of gene delivery applications are taking advantage of the evolutionary experience of the viruses, particles which have optimised systems to ensure the efficient delivery of their genetic material to the nucleus of the target cell.

#### 1.4.2.1 Adenovirus

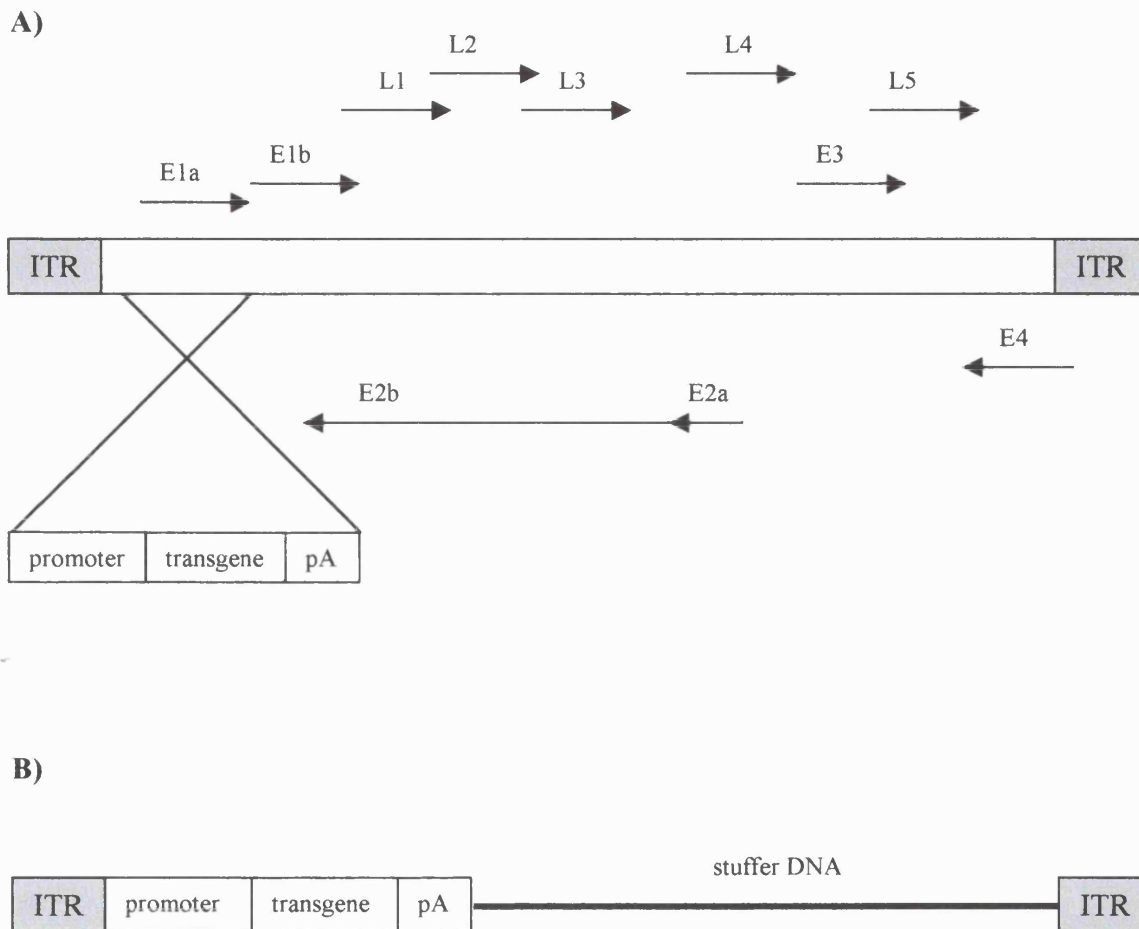
Adenoviruses are non-enveloped double-stranded DNA viruses with a genome of approximately 35kb. Viral capsid proteins facilitate the entry of the virus into the cell by interacting with a cellular receptor (coxsackie and adenovirus receptor, Bergelson *et al.*, 1997) and integrin proteins (Wickham *et al.*, 1993). The virus then enters the cell by receptor mediated endocytosis and migrates to the nucleus where viral transcription and replication begins.

Transcription of the adenoviral genome follows an ordered sequence, with the expression of the early genes (E) involved in the replication of the viral genome, followed by the expression of the late genes (L) involved in the synthesis of the viral capsid.

The expression of E1A gene products results in the transactivation of all the other early genes (Richardson and Westphal, 1981). It was therefore anticipated that deletion of E1A would render the virus replication incompetent and suitable as a vector for gene transfer. The E3 region was also deleted as it is not necessary for replication *in vitro*, although it plays an important role *in vivo* in the modulation of antigen presentation. E1 deleted viruses can be generated and amplified in 293 cells, a human embryonic kidney cell line which constitutively expresses the E1 proteins (Graham *et al.*, 1977). However, these viruses were found not to be completely replication incompetent as endogenous cellular factors such as NF-IL6 can transactivate adenoviral genes with E1A responsive promoters (Spergel and Chen-Kiang, 1991; Spergel *et al.*, 1992). These first generation adenoviral vectors were used to deliver a number of reporter and therapeutic genes *in vitro* and *in vivo* (Akli *et al.*, 1993; Horellou *et al.*, 1994; Lusky *et al.*, 1998). However, these vectors proved unsuitable as they still

produced a number of viral gene products and elicited a strong immune response from the host (Yang *et al.*, 1994a; Lusky *et al.*, 1998). Further developments of the system led to the additional deletion or inactivation of either E2a or E2b, which code for a DNA binding protein and DNA polymerase respectively (Yang *et al.*, 1994b; Amalfitano *et al.*, 1998). These deletions significantly reduced the titres achievable and made the propagation process more cumbersome but these second generation vectors were found to facilitate longer vector persistence even when the vector encoded a foreign antigen (Hu *et al.*, 1999). Third generation viruses included the deletion of the E4 region of the genome. The genomes of these vectors were found to persist longer term *in vivo*, for up to 84 days in one study (Dedieu *et al.*, 1997) although the time course of transgene expression was considerably more short lived. This shut off of transgene transcription was found to be characteristic of E4 deleted viruses (Kaplan *et al.*, 1997), suggesting that the design of these vectors might have to be a compromise between virus attenuation and reduced levels of transgene expression.

Further developments of adenovirus technology have led to the generation of “gutless” vectors (Fisher *et al.*, 1996; Kumar-Singh and Chamberlain, 1996; Kochanek *et al.*, 1996). These vectors are deleted for all viral genes and only retain the packaging and replication signals. A replication incompetent adenovirus is used as a helper to provide the necessary deleted gene products *in trans*. Viral stocks are prepared by transfection and superinfection of a complementing cell line and the plasmid vector is packaged into virions. The helper virus can then be separated from the vector by gradient centrifugation or eliminated by Cre-*lox* recombination to remove the packaging signal from the helper genome (Hardy *et al.*, 1997). The deletion of all the viral genes greatly expands the transgene capacity of these vectors, from approximately 10kb in third generation vectors to over 30kb in gutless vectors. Although these gutless vectors do not elicit a strong cytotoxic response and can sustain transgene expression in the long term (Schiedner *et al.*, 1998), removal of the helper virus is not complete. A contamination level of about 1% has been estimated by Southern blot analysis (Chen *et al.*, 1997). The structure of the adenovirus genome and the transcriptional units which can be deleted or inactivated in vector design is shown in figure 1.4.2.1.1. Table I summarises the main differences between the four generations of adenovirus vectors.



**Figure 1.4.2.1.1 Schematic diagram of the transcriptional units of adenovirus and the engineering of the viral genome which generates adenovirus vectors**

A) The genome of adenovirus. Both the negative and positive strands code for viral proteins. The E1, E3 and late genes are all transcribed from the negative strand whereas E2 and E4 are transcribed from the positive strand. In order to generate replication defective adenovirus vectors, genes are deleted from E1, E2, E3 or E4. The inserted transgene cassette is here shown inserted into E1 by homologous recombination. B) A gutless adenovirus vector consists of only the ITRs (which code for packaging and replication signals), the transgene and its regulatory sequences and stuffer DNA to enable efficient packaging.

Vector	Genes deleted/ inactivated	Cloning capacity	Advantages	Disadvantages
First generation	E1 (and E3)	7.5kb		Residual replication despite lack of E1
Second generation	E1 and E2a or E2b (and E3)	9kb	Severe inhibition of viral protein synthesis	Incomplete inhibition of ts E2a at 37°C
			Reduced immune response	
			Increased genome persistence and transgene expression	
Third generation	E1 and E4 (and E3)	10kb	Improvement in genome persistence	Reduced expression of transgene
			Reduced leakiness of viral protein expression	
Gutless	All viral genes	35kb	Expresses no viral genes	Requires helper virus
			Long term persistence	
			Large cloning capacity	

**Table I Advantages and disadvantages of adenoviral vectors**

The advantages and disadvantages of the different generation adenovirus vectors are listed. The deletion of E3 is in brackets as some groups prefer to leave this gene present in order to assist in the evasion of the host's immune response. Table adapted from Hermens and Verhaagen, 1998 and Benihoud *et al.*, 1999.

Adenovirus DNA remains episomal in the host cell nucleus. Whilst this has the advantage of not presenting any risk of insertional mutagenesis, it does suggest that the DNA would be gradually degraded by cellular nucleases, leading to the eventual clearance of adenoviral genomes from the cell. However, transgene expression has been reported to persist for several months in neurons, albeit at a significantly reduced level from that initially achieved (Davidson *et al.*, 1993).

From the foregoing discussion, it can be seen that the relatively short term expression achieved from adenovirus vectors could be attributable to a number of factors, although it appears likely that the immune response of the host is paramount. This conclusion is supported by the observation that transgene expression can be detected for considerably longer in young animals with underdeveloped immune systems or in those which are deficient in T-lymphocytes or are immunosuppressed (Yang *et al.*, 1994a; Kass-Eisler *et al.*, 1994).

The E3 transcription unit of adenovirus encodes a number of proteins which enable the virus to avoid immunological surveillance by the host. For this reason, several of the later generation adenoviruses do not have this region deleted. The E3 19kDa glycoprotein (19K) interacts with the peptide binding domain of MHC class I molecules. An ER retention signal on 19K causes these complexes to be retained in the ER, thereby preventing presentation of antigens on the cell surface (Andersson *et al.*, 1985). Other E3 encoded proteins which assist in the subversion of the host's immune response include 14.7K and 10.4K. These proteins act either independently or as a heterodimer to prevent tumour necrosis factor (TNF) cytotoxicity (Chen *et al.*, 1998), down-regulate Fas receptor levels and block the caspase-8 apoptotic pathway (Tollefson *et al.*, 1998).

It has been suggested that gene expression from the adenoviral backbone is not necessary for cytotoxic T-lymphocyte (CTL) dependent lysis of infected cells (Kafri *et al.*, 1998). These authors observed that UV-inactivated virus from which no gene expression was detectable was still capable of causing target cell lysis. This would indicate that the adenoviral capsid proteins are themselves cytotoxic, a suggestion which has been disputed by other laboratories (Jooss *et al.*, 1998). However, a consideration of the internalisation step in the adenovirus lifecycle might provide an

explanation which would tend to support the former result. Internalisation leads to a rapid activation of the Raf/MAPK signalling pathway which then induces interleukin 8 expression. It has been proposed that this could recruit CTLs to vector transduced cells (Bruder and Kovesdi, 1997). The fact that the activation of this pathway occurs within 20 minutes of infection has led some to conclude that adenovirus is fatally flawed as a gene transfer vector (Kafri *et al.*, 1998). If indeed it does transpire that the initial events of infection are all that is necessary to elicit a full CTL response, then it would be hard to argue with this conclusion.

The potential advantages of adenovirus vectors are that they can be grown to very high titres (approximately  $10^{11}$  pfu/ml), they do not integrate yet their genomes are relatively stable and they have a broad host range, infecting both dividing and non-dividing cells. Whilst the vector might provide a suitable gene transfer vehicle for *in vitro* studies, the immunogenicity of the vector system currently remains an obstacle to successful and widespread *in vivo* use.

#### **1.4.2.2 Adeno-associated virus (AAV)**

AAV is a member of the parvovirus family. The virus is non-enveloped and has a 4.6kb single stranded DNA genome. In order for productive infection to occur, a helper virus is required to provide essential functions *in trans*. These helper functions are usually provided by adenovirus or herpesvirus. AAV is a simple virus consisting of only two open reading frames (ORFs), one encoding genes necessary for replication (*rep*) and the other encoding genes necessary for the formation of the viral capsid (*cap*). The AAV genome is flanked by inverted terminal repeat (ITR) sequences which contain the viral origin of replication and 145 nucleotide palindromic sequences. These palindromes facilitate the formation of 'hairpin' structures which are important in the virus replication cycle and also confer resistance to exonuclease activity, stabilising the viral genome.

AAV is unique among eukaryotic viruses in that it preferentially integrates into the host genome at a specific site on the long arm of chromosome 19 (Kotin *et al.*, 1992). Sequencing of this site precluded the possibility that the integration was a result

of homologous recombination but identified a 33 base pair sequence which specifically interacted with an AAV *rep* protein (Linden *et al.*, 1996).

AAV was originally identified as a contaminant of laboratory stocks of adenovirus. Whilst it is often found in humans with adenovirus infections, AAV does not modify the severity or symptoms of the infection and as such is considered to be non-pathogenic. This suggested that AAV might be suitable for development as a gene transfer vector.

Recombinant AAV vectors are constructed by cotransfecting a plasmid containing the transgene cassette, the AAV ITRs and packaging sequences with a plasmid expressing the AAV *rep* and *cap* proteins under the control of the adenovirus ITRs. The transfected cells are then superinfected with a helper virus, usually adenovirus. Following harvesting, a mixed population of recombinant AAV and helper virus is obtained but the latter can be removed by differential centrifugation or heat treatment. The process of generating recombinant AAV is schematically illustrated in figure 1.4.2.2.1. Since the *rep* proteins are not expressed the recombinant AAV does not have the capacity for site specific integration. However, experiments on HeLa cells have determined that the ITRs are necessary and sufficient for efficient integration to occur, with rAAV integrating at loci on chromosomes 1, 2, 7 and 17 (Yang *et al.*, 1997).

Recombinant AAV mediated infection of neurons has been documented and reported to cause negligible cytopathic effects (McCown *et al.*, 1996; Du *et al.*, 1996). In these papers, expression of the transgene was observed for several months. It was not clear whether the eventual loss of expression reflected a lack of integration or whether it was a result of promoter downregulation. AAV mediated expression of tyrosine hydroxylase (TH), glial derived neurotrophic factor (GDNF) or TH and GTP-cyclohydrolase in the rat striatum *in vivo* has led to behavioural recovery in animal models of Parkinson's disease (Kaplitt *et al.*, 1994b; Mandel *et al.*, 1997; Mandel *et al.*, 1999b; Szczypka *et al.*, 1999). The AAV mediated delivery of TH and aromatic amino acid decarboxylase (AADC) to primate striatum *in vivo* has also been reported. In this study, immunoreactivity for the vector-encoded TH was detectable for up to 134 days post injection but the observed biochemical and behavioural differences between the AAV/TH-injected animals and a control group were not statistically significant (During

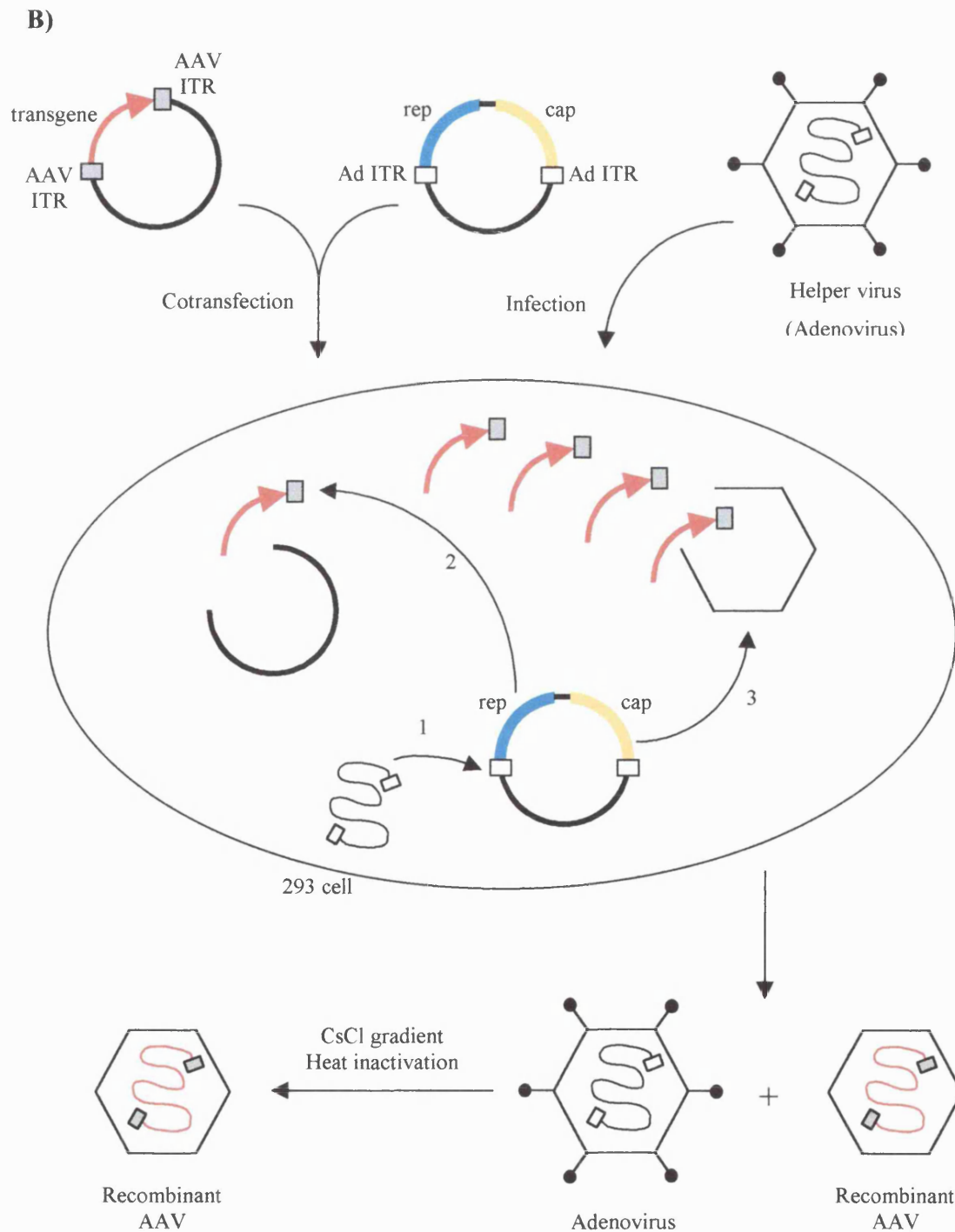
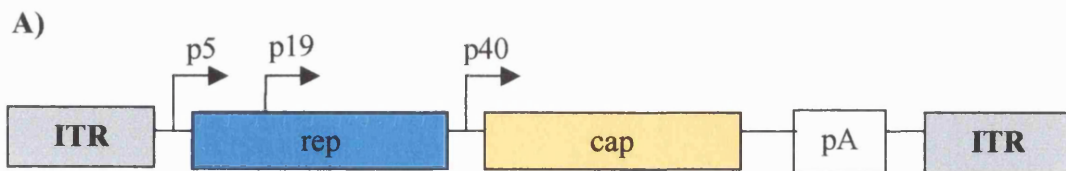


*et al.*, 1998). AAV has also been successfully used to deliver NGF to the rat medial septum, protecting these cholinergic neurons from death following axotomy in a biochemical animal model of Alzheimer's disease (Mandel *et al.*, 1999a). The potential for very long term expression from rAAV has been demonstrated by studies assessing gene transfer to the skeletal muscle of mice (Xiao *et al.*, 1996). This paper reported transgene expression for at least 18 months post injection.

The major advantage of AAV as a gene delivery vector is that it is non-toxic and non-immunogenic. However, the production method for rAAV is very cumbersome, involving the transfection of two plasmids and superinfection with helper virus. This cannot easily be scaled up and low transfection efficiencies severely limit the titres achievable. Strategies to increase titres and maintain consistency between preparations include the use of alternative helper functions, either a plasmid encoding the necessary adenovirus proteins (Xiao *et al.*, 1998) or a herpesvirus expressing *rep* and *cap* (Conway *et al.*, 1997; Conway *et al.*, 1999). Attempts to construct stable cell lines expressing *rep* and *cap* have been hindered by the inherent toxicity of the *rep* proteins. Where such cell lines have been reported they have often proven to yield lower titres of recombinant AAV than the cotransfection method (Clark *et al.*, 1995; Clark *et al.*, 1996). Another limitation with AAV as a gene delivery vector is that the single strand genome obviously requires second strand synthesis before the transgene can be expressed. This is rate-limiting in all cell types (Fisher *et al.*, 1996; Ferrari *et al.*, 1996) and might be particularly inefficient in cells which have little or no DNA replication. Furthermore, AAV packaging limits the cloning capacity of this vector system to about 4kb.

#### **Figure 1.4.2.2.1      Production of recombinant adeno-associated virus**

Map of the AAV genome. The *rep* and *cap* genes are shown flanked by the ITRs. The three promoters which control the expression of *rep* and *cap* are indicated. B) Production of a recombinant AAV vector. A plasmid vector containing the transgene inserted between the AAV ITRs is cotransfected with a plasmid expressing the AAV *rep* and *cap* genes under the control of adenovirus ITRs. The transfected cells are then superinfected with a helper virus (usually adenovirus). In the cell, the adenoviral gene products transactivate the adenovirus ITRs and enhance the production of *rep* and *cap* (1). *Rep* gene products then act to transactivate the AAV ITRs causing replication of the transgene (2). *Cap* gene products form the AAV capsid and package the newly synthesised AAV genomes (3). Harvesting yields a mixed population of recombinant AAV and helper virus. Helper virus is then removed by centrifugation on a CsCl gradient and heat inactivation. Figure adapted from Hermens and Verhaagen, 1998



#### 1.4.2.3 Retroviruses

Retroviruses are small RNA viruses which replicate through a DNA intermediate. All retroviruses infect host cells through a specific interaction with a viral envelope protein and a cell surface receptor. Once inside the cell, the RNA genome is transcribed into dsDNA using reverse transcriptase contained within the viral particle. The DNA is then transported to the nuclear membrane. At mitosis, the integrity of the host cell nuclear matrix is compromised, enabling the viral DNA to integrate into the host genome. Integration is mediated by the virally encoded integrase enzyme. Within the host genome, integration can occur at a large number of chromosomal locations with no apparent preference or specificity. This integrated 'provirus' is then replicated with the host DNA and is passed on to all progeny cells. After integration, the viral LTR promoter at the 5' end of the genome is usually active and directs the synthesis of an unspliced copy of the viral genome, which terminates at the 3' LTR.

The retroviral genome includes the genes *gag*, *pol* and *env* which code for core proteins, replication enzymes and envelope glycoproteins, respectively. The unspliced copy of the viral genome serves several functions. Firstly, it is the mRNA for the enzymes reverse transcriptase and integrase and for all the *gag* proteins which comprise the bulk of the viral particle. Secondly, it is a precursor RNA, generating a spliced mRNA for the *env* protein. Lastly, it is a genomic RNA as it encodes the encapsidation sequence ( $\Psi$ ), facilitating its packaging into viral particles. These particles are then budded from the host cell through the *env*-coated cell membrane.

The fact that retroviruses integrate into the host genome circumvents the problems of transient expression which are commonly encountered with other gene delivery vectors. This has led to them being a popular choice for gene transfer. Recombinant retroviruses contain only the encapsidation sequence and the terminal repeat sequences, the virion components are provided *in trans* by a packaging cell line. Recombinant retroviruses based on Moloney murine leukaemia virus (MMLV) have been successfully used in many applications *in vitro* and *in vivo* and are the most commonly used gene delivery system currently in clinical trials. However, like the majority of retroviruses, MMLV requires the breakdown of the nuclear matrix at mitosis in order to access the cellular genome. This renders this virus unsuitable for direct gene

delivery to non-dividing cells such as neurons, although MMLV has been used extensively to deliver genes to cells in culture which are then grafted into the brain. Examples of this approach include the transplant of retrovirally transduced fibroblasts expressing TH which were found to cause behavioural recovery in an animal model of Parkinson's disease (Wolff *et al.*, 1989; Horellou *et al.*, 1990). Similarly, fibroblasts transduced to express NGF rescued axotomised cholinergic neurons in a biochemical animal model of Alzheimer's disease (Rosenberg *et al.*, 1988). However, although the grafted cells are able to survive long term, the transgene is usually inactivated over time (Palmer *et al.*, 1991). For the purposes of this thesis, MMLV is inherently unsuitable as high efficiency delivery of genes to neurons is required.

Lentiviruses are a subgroup of the retroviruses. The basic life cycle is as described above for a generic retrovirus, although the genomes of lentiviruses are slightly more complicated, encoding several additional proteins. For gene delivery to the nervous system, lentiviral vectors have a major advantage over murine retroviruses in that they do not require the breakdown of the host cell nuclear matrix in order to integrate. This ability to stably infect non-dividing cells is imparted by two virion proteins, matrix (MA) and virion protein R (*vpr*). These proteins interact with the nuclear import machinery to facilitate active transport of the lentivirus preintegration complex through the nuclear pores (Gallay *et al.*, 1995a; Gallay *et al.*, 1995b; Gallay *et al.*, 1996).

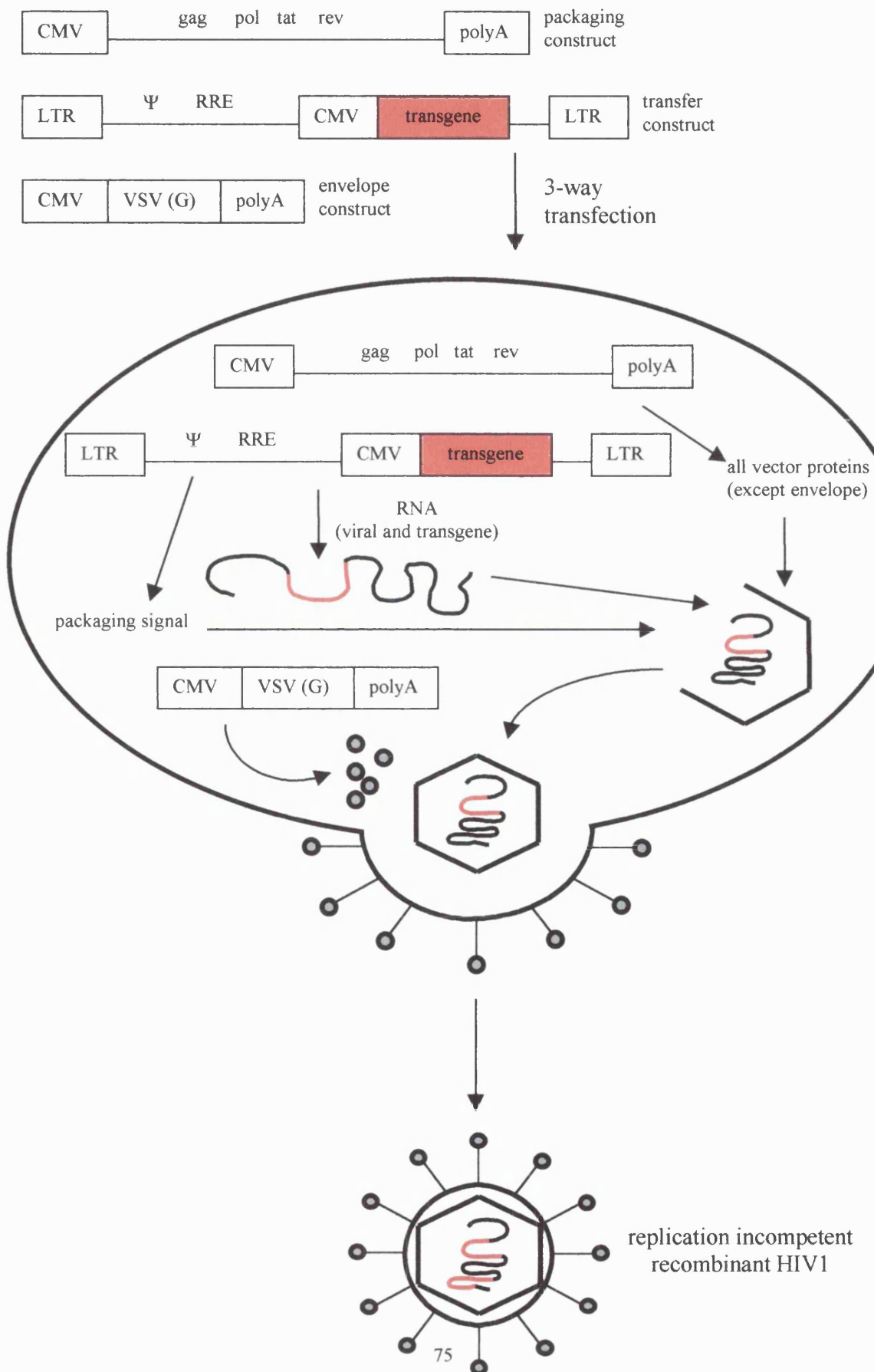
Lentiviral vectors have only relatively recently been described and are currently based on human immunodeficiency virus 1 (HIV 1) (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b) or equine infectious anaemia virus (Mitrophanous *et al.*, 1999). Herpes simplex virus, adenovirus and adeno associated virus, the other viruses often used as gene transfer vectors, are all common human pathogens which cause minimal or no disease symptoms in the vast majority of infections. In contrast, the pathogenicity of HIV 1 makes the use of this virus as a vector more controversial. In an attempt to minimise the potential of a recombination event which could result in the production of a wild type virus, the vector production system is slightly convoluted and involves the use of multiple plasmids. The system is designed to completely segregate the *trans*-

acting sequences encoding the viral proteins away from the *cis*-acting sequences involved in the transfer of the viral genome.

Production of lentiviral vectors is based on a transient expression system. A transfer vector, a packaging vector and an envelope vector are cotransfected into mammalian cells. The transfer vector contains retroviral *cis*-acting elements and the gene of interest, usually driven by a CMV promoter. These elements are insufficient to allow the cytoplasmic export of full length vector transcripts in the absence of *rev* (regulator virion proteins) and *tat* (transactivator protein), which are encoded on a separate plasmid, the packaging construct. The packaging construct is identical to the HIV 1 provirus except it has the reading frames of *env* and *vpu* disrupted and the lentiviral LTRs have been replaced with a CMV promoter and insulin polyadenylation signal. The result of this is that the packaging construct is deficient for the *cis* acting sequences necessary for packaging ( $\Psi$ ), reverse transcription and integration, the former having been deleted and the latter two being unable to be transcribed. The final plasmid, the envelope vector, can be varied to target a particular host. This is one of the main advantages of all retroviral vectors. A commonly used envelope is that from vesicular stomatitis virus glycoprotein G (VSV G). Pseudotyping in this way means that the infectivity of the recombinant virus is not limited to CD4 expressing T lymphocytes, the usual target cells of HIV 1. A schematic diagram of the lentivirus vector system is shown in figure 1.4.2.3.1.

#### **Figure 1.4.2.3.1 Construction of a recombinant lentiviral vector**

The packaging construct, the transfer construct and the envelope construct are cotransfected, usually into 293 cells. The packaging construct contains all the *trans*-acting sequences which encode the viral proteins. The transfer construct contains all the *cis*-acting sequences which are required in the transfer of the viral genome to the target cell. Sequences encoded by the transfer construct are the only ones transferred to the recombinant virus. RRE stands for rev response element and is required for controlling the splicing of the viral genome. The envelope construct provides the envelope proteins, pseudotyping the virus to target a particular cell type.





Although still a relatively new technology, it has been shown that lentivirus vectors can sustain expression of a reporter gene under the control of the CMV promoter for over 6 months in striatal and hippocampal neurons *in vivo* (Blomer *et al.*, 1997). The authors compared adenovirus, adeno-associated virus, MMLV and lentivirus vectors and reported that only the latter was able to demonstrate sustained transgene expression over time. It was proposed that lentiviruses allow insertion into chromatin domains which are permissive for long term expression. There is no expression of viral proteins to elicit an immune response and the integrated transgene becomes, in effect, a cellular gene. Work from the same laboratory has also demonstrated the long term delivery of physiologically relevant genes to the CNS. It was reported that delivery of antiapoptotic factors (Bcl-xL) and neurotrophins (NGF) could protect cholinergic neurons from death following axotomy (Blomer *et al.*, 1998).

Whilst the integration of retroviral genomes offers the potential for stable long term transgene expression, the risk of insertional mutagenesis must also be considered. This issue has been addressed by Miyoshi and colleagues who reported the construction of self-inactivating lentivirus vectors (Miyoshi *et al.*, 1998). These vectors have the viral enhancer and promoter sequences deleted, precluding the possibility that integration into the host genome will cause the activation of a nearby cellular oncogene. Whilst this approach addresses the problem of insertional activation, there will be no way of overcoming the risk of insertional inactivation of, for example, a cellular tumour suppressor gene. Another potential limitation of this system is that lentiviral vectors can only package 7-10kb of exogenous DNA.

The long term transgene expression, stable integration and lack of expression of viral proteins are likely to make lentiviral based vectors an attractive tool for future gene delivery applications. Currently, however, the vectors are considered high risk and work must be carried out under levels of high containment.

#### **1.4.2.4 Herpes simplex virus 1 (HSV1)**

HSV1 has often been suggested as a gene delivery vector for the nervous system because it has a natural propensity to infect neurons. Furthermore, the fact that HSV1 does not integrate into the host genome but is capable of establishing a latent infection

has suggested that novel methods of obtaining long term gene expression from such a viral vector could be investigated. In addition, HSV1 has a very large genome, meaning that it is not subject to the packaging constraints imposed by some other viral vector systems. For these reasons, we and others have chosen to develop HSV1 as a vector for delivering genes to neurons.

## **1.5 The biology of HSV1**

The following sections are dedicated to a discussion of the biology and lifecycle of HSV1 and the adaptations which must be made to this lifecycle in order to generate an efficient vector system.

### **1.5.1 The structure of the virion**

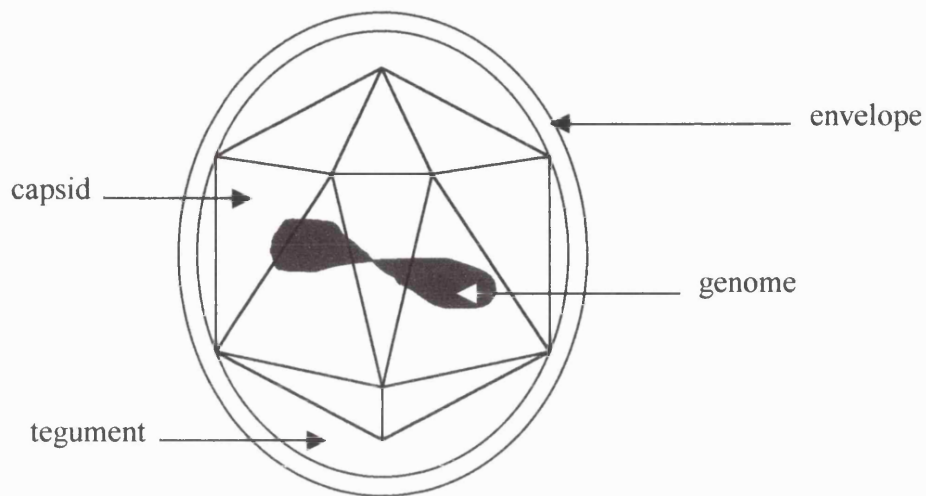
The core of an HSV1 virion is electron opaque and contains the viral genome. This core is surrounded by an icosahedral capsid and an outer lipid envelope. Between the capsid and the lipid envelope is the amorphous tegument layer. A schematic diagram of an HSV1 virion is shown in figure 1.5.1.1A.

A number of proteins are associated with the virion. The envelope contains a number of glycoproteins (named gB to gM) which are involved in attachment and penetration and the tegument houses several proteins which assist in the early stages of infection. The specific roles of a number of these virion proteins will be discussed in more detail later.

### **1.5.2 The viral genome**

HSV1 is a double stranded DNA virus. The linear genome of approximately 152kb encodes at least 80 genes. The genome is composed of long and short unique segments flanked by terminal repeat regions. The unique segments can invert relative to each other to generate four possible isoforms. The structure of one of these isoforms is shown in figure 1.5.1.1B.

A)



B)



**Figure 1.5.1.1 Schematic diagram of the HSV1 virion and genome**

A) The structure of the HSV1 virion is shown with the DNA-containing capsid, tegument and glycoprotein-containing envelope indicated. B) The organisation of the HSV1 genome, showing the unique long and short regions ( $U_L$  and  $U_S$  respectively) flanked by the terminal and internal repeats (TR and IR respectively). The regions marked 'a' contain sequences required for packaging. Figure adapted from Fink *et al.*, 1996.

### 1.5.3 Cell attachment

The exact details of the mechanism by which HSV1 attaches and gains entry to cells have not been elucidated. However, heparan sulphate has been identified as an important factor in the binding of HSV1 to the cell surface (WuDunn and Spear, 1989; Shieh *et al.*, 1992). Evidence to suggest that heparan sulphate is the primary receptor for HSV1 includes the observation that heparin is able to inhibit virus attachment in a dose-dependent manner, presumably by competing with the virus for the heparan sulphate receptor (Shieh *et al.*, 1992). Cell lines which are unable to form the repeating disaccharide unit of heparan sulphate have been shown to be 85% resistant to HSV1 infection, lending further support to the suggestion that heparan sulphate is the primary, but perhaps not the sole, receptor for HSV1 (Gruenheid *et al.*, 1993). Proposed candidates for secondary or co-receptors have included the fibroblast growth factor receptor (FGFR) (Baird *et al.*, 1990) although this suggestion has been disputed by other laboratories who were unable to substantiate the finding (Shieh and Spear, 1991; Muggeridge *et al.*, 1992).

An understanding of the envelope glycoproteins which are involved in binding to heparan sulphate has come from studies of viral mutants lacking functional versions of one or more of the twelve known glycoproteins. Four envelope glycoproteins (gB, gD, gH and gL) and one non-envelope glycoprotein (gK) have been found to be essential for productive infection in cell culture (Desai *et al.*, 1988; Hutchinson *et al.*, 1992; Fuller and Lee, 1992; Hutchinson and Johnson, 1995).

### 1.5.4 Cell penetration

The identification of a cellular receptor which facilitates virus penetration came from a series of experiments carried out by Montgomery and co-workers. These authors generated a cDNA library from a cell line known to be permissive for HSV1 infection and transiently transfected plasmid clones from this library into cells which were resistant to HSV1 entry. A receptor was found which could confer susceptibility onto these previously resistant cells. The novel receptor was named Herpes Virus Entry Mediator (HVEM) and it was found to be a member of the TNF/NGF receptor family (Montgomery *et al.*, 1996). The authors generated an anti-serum to HVEM and were

able to demonstrate that binding of HSV1 to cells was not affected, suggesting that HVEM was involved in the penetration of the plasma membrane. Transfection of resistant cells with the HVEM cDNA could not confer susceptibility to viruses with mutations in gD, although these viruses are known to be able to infect cells. This suggests that, like heparan sulphate, HVEM may be only one of a number of receptors important in mediating attachment and entry.

Again, viral mutants have been employed in determining which glycoproteins are required for penetration. gD, gH and gL have all been implicated, the former acting individually and the latter two acting in concert (Fuller and Lee, 1992; Roop *et al.*, 1993). The study discussed above would suggest that in the case of gD at least, the role of the glycoprotein is to interact with HVEM.

Following penetration and internalisation, several of the tegument proteins have an important function. These include the virion host shut off (*vhs*) protein which indiscriminately degrades mRNA and so causes an early shut down of host protein synthesis (Kwong and Frenkel, 1987). Virus capsids are transported to the nucleus by retrograde axonal transport where the viral DNA and at least some tegument proteins enter the nucleoplasm by an unknown mechanism. Here the course of the HSV1 infection depends on whether the virus enters the lytic cycle or establishes a latent infection.

### 1.5.5 The lytic lifecycle

In order for the efficient initiation of the lytic cycle, the tegument protein, virion protein 16 (VP16)<sup>1</sup> must interact with cellular factors to transactivate the promoters of the first class of viral genes to be expressed, the immediate early (IE) or  $\alpha$  genes. VP16 cannot bind DNA directly so it is dependent on the cellular POU domain protein Oct-1 and at least one other cellular factor, known as host cell factor (HCF) to form a multicomponent complex on the TAATGARAT motifs which are present in all the IE gene promoters (Gaffney *et al.*, 1985). HCF has an important role in transporting VP16 to the nucleus (La Boissiere *et al.*, 1999), Oct-1 binds directly to the TAAT region of

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<sup>1</sup> The virion protein VP16 is also referred to as  $\alpha$ -transinducing factor ( $\alpha$ -TIF) or vmw 65 (as determined by the molecular weight). However, throughout this thesis the protein will be referred to as VP16.

the motif, and the recruitment of VP16 is dependent on the presence of the GARAT half of the sequence (where R is a purine) (Gerster and Roeder, 1988; O'Hare *et al.*, 1988). VP16 is known to contain a very potent C-terminal transactivation domain (Sadowski *et al.*, 1988). The location of the TAATGARAT motifs just upstream of the TATA box sites of the IE gene promoters and the observation that VP16 can interact directly with transcription factor TFIID suggests that VP16 acts directly to activate the basal transcription machinery (Klemm *et al.*, 1995).

Following this transactivation, the lytic gene cascade ensues. This cascade is well-ordered and tightly regulated and dictates the controlled expression of three classes of genes; the IE or  $\alpha$  genes already mentioned, the early (E) or  $\beta$  genes and the late (L) or  $\gamma$  genes (Honess and Roizman, 1974). A schematic representation of this gene cascade is shown in figure 1.5.5.1.

There are five IE genes; infected cell polypeptides (ICP) 0, 4, 22, 27 and 47. ICP0 is not essential for a productive infection but its deletion has been shown to significantly impair viral replication, especially at low multiplicity of infection (MOI) (Chen and Silverstein, 1992; Cai and Schaffer, 1992). Transient transfection studies have shown that ICP0 is a promiscuous transactivator of almost any target promoter, and that this transactivation is enhanced in the presence of ICP4 (Everett, 1984; Everett, 1987). ICP0 is unable to bind DNA directly so the exact mechanism by which it affords this transactivation is unclear. However, it has become apparent that ICP0 acts at or before the initiation of mRNA synthesis (Jordan and Schaffer, 1997). There has been much interest in ICP0, not least because its position in the genome (ICP0 is transcribed antisense to LAT, see later) suggests that it might have a role in reactivation from latency. This might give functional relevance to the observations that ICP0 can transactivate IE gene promoters in the absence of virion proteins such as VP16 and is required during infections at low MOI, both situations which are not dissimilar from that occurring during the reactivation from latency.

ICP4 is an essential regulatory IE gene and is the major transcriptional regulator of HSV1 (DeLuca *et al.*, 1985). ICP4 is a DNA binding protein, recognising the consensus site RTCGTCNNYNYSG, where R is purine, Y is pyrimidine, S is C or G, and N is any base (DiDonato *et al.*, 1991). ICP4 is able to transactivate E and L genes

and downregulate the expression of some IE genes, especially ICP0 and itself (Roberts *et al.*, 1988). ICP4 has also been demonstrated to repress the activity of the latency promoters, either on its own or in combination with ICP0 (Batchelor and O'Hare, 1990; Goins *et al.*, 1994). The precise mechanism of action of ICP4 is unclear but the protein has been shown to interact directly with several components of the basal transcription machinery (Smith *et al.*, 1993; Gu *et al.*, 1995; Kuddus *et al.*, 1995; Carrozza and DeLuca, 1996).

ICP27 is an essential regulatory IE gene (Sacks *et al.*, 1985) encoding a nuclear phosphoprotein which performs a number of diverse regulatory functions. These include repression of IE and E genes, activation of late genes and selection of transcriptional termination sites (McLauchlan *et al.*, 1989; McCarthy *et al.*, 1989). ICP27 also contributes to the shut off of host protein synthesis seen during productive infection, through impairment of host cell pre-mRNA splicing (Hardy and Sandri-Goldin, 1994; Hardwicke and Sandri-Goldin, 1994).

ICP22 is non-essential for viral growth but has been demonstrated to promote efficient late gene expression in a cell type dependent manner (Sears *et al.*, 1985). ICP22 has also been implicated in the production of an aberrantly phosphorylated form of cellular RNA polymerase II (Rice *et al.*, 1995; Long *et al.*, 1999).

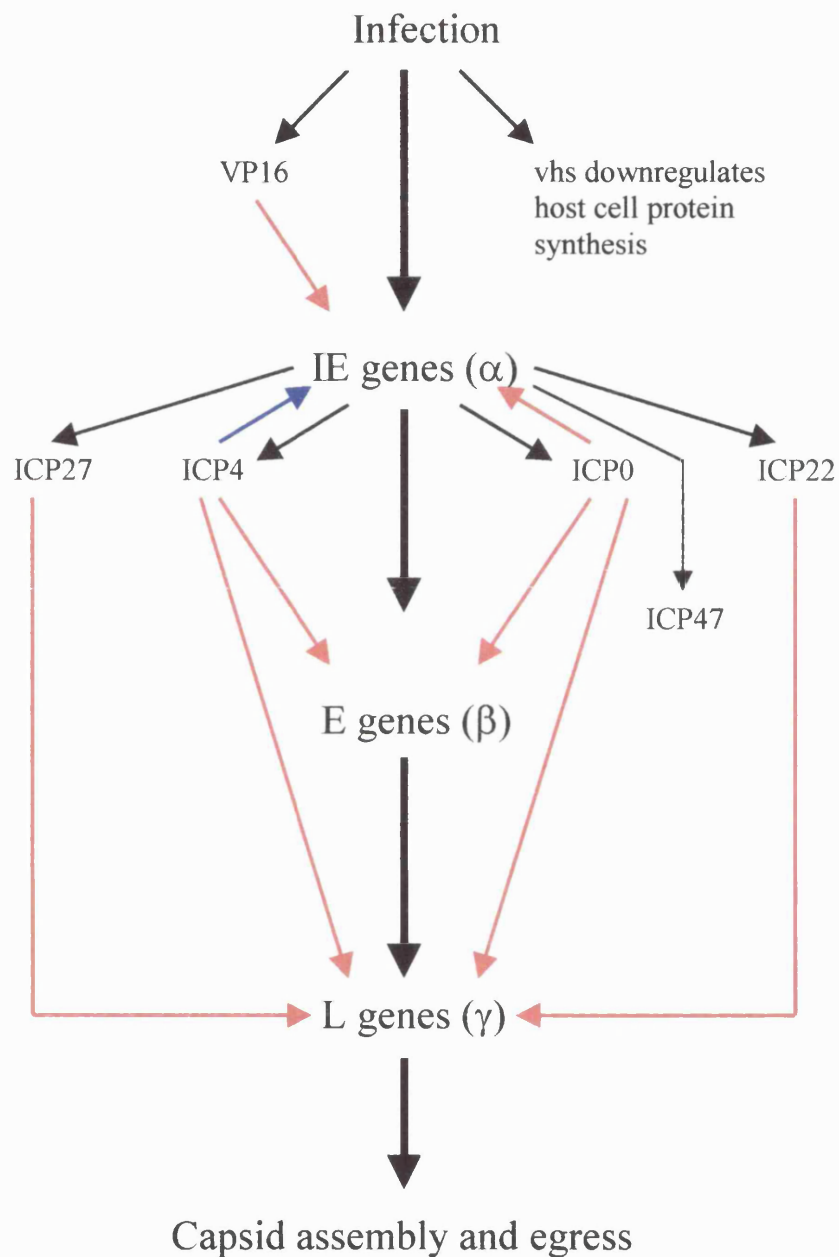
ICP47 is non-essential for replication and is not a regulatory IE gene (Mavromara-Nazos *et al.*, 1986). However, it is known to inhibit antigen presentation to CD8<sup>+</sup> T lymphocytes, possibly providing a mechanism by which HSV1 escapes immune surveillance by the host (York *et al.*, 1994). By constructing a cell line with a regulatable promoter driving expression of ICP47, it was shown that HSV1 modulates antigen processing at the level of the transporters of antigen processing (TAP) (Hill *et al.*, 1995). Processing of viral proteins for recognition by cytotoxic T-lymphocytes normally involves degradation of the proteins in the cytosol of an infected cell followed by transport of the resulting peptides into the endoplasmic reticulum (ER) by the TAP. In the ICP47-expressing cell line, it was shown that cytosolic ICP47 bound with high affinity to the TAP and blocked the binding of antigenic peptides. Due to the lack of translocated peptides inside the endoplasmic reticulum, MHC class I molecules fail to

assemble and therefore MHC-peptide complexes do not reach the cell surface for immune recognition.

Following the synthesis of the IE genes, the E genes are transcribed. The E gene products are primarily involved in viral DNA synthesis and encode proteins such as DNA binding proteins, polymerases and thymidine kinase. Viral DNA synthesis occurs by a rolling circle mechanism, forming head-to-tail concatemers of UL and US separated by the repeat regions (Jacob *et al.*, 1979).

Expression of the L genes is activated by the IE genes only after viral DNA synthesis has occurred. The L genes encode the structural proteins of the capsid, tegument and envelope. Following the expression of the L genes, the viral DNA concatemers are cleaved into genome length units and packaged into the capsids. The cleavage and packaging signal derives from 'a' sequences that lie between the repeat regions of the DNA concatemer (see figure 1.5.1.1). The capsids then bud through areas of the nuclear envelope that have been modified with viral glycoproteins, thus forming the viral envelope. The newly synthesised virions then pass through the ER and cytoplasm and into the extracellular space. The observation of partially enveloped virions at cytoplasmic membranes has led to the suggestion that some virions may lose and re-acquire their envelopes during transit through the ER (reviewed in Roizman and Sears, 1996). The first newly formed capsids appear in the nucleus within six hours of infection and the entire lytic cycle takes approximately ten hours.





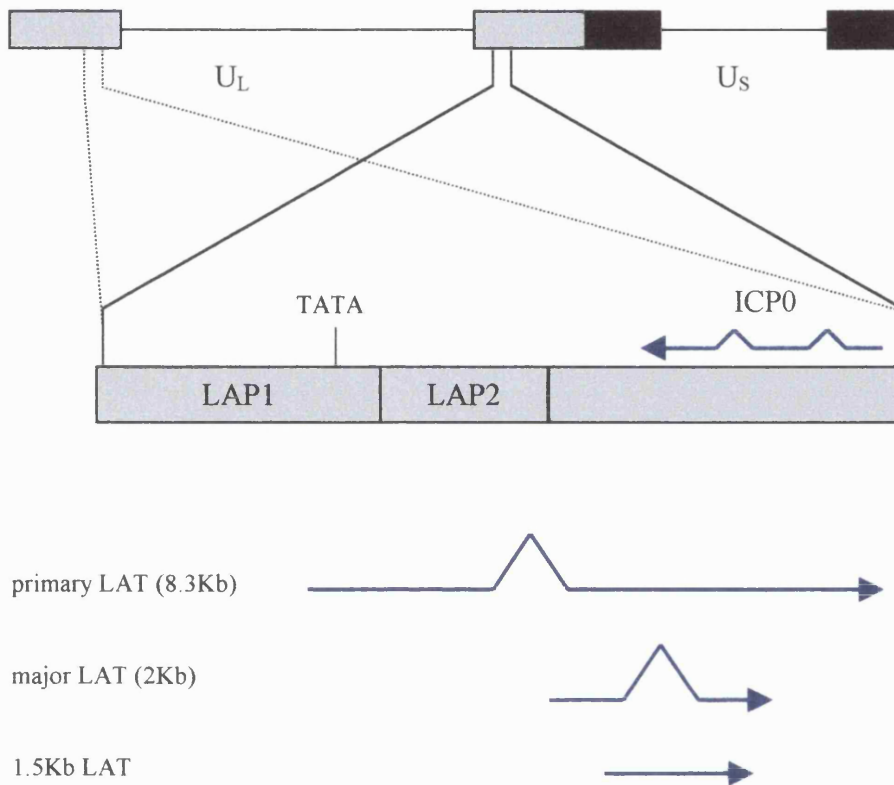
**Figure 1.5.5.1 The HSV1 lytic gene regulatory cascade**

The virion protein VP16 interacts with Oct-1 and HCF to transactivate the IE genes. Expression of IE genes is required for the timely expression of E and L genes. Known positive regulatory effects are indicated with red arrows whereas repressive effects are indicated with blue arrows. There are a number of interactions between the regulatory IE genes which are also known to mediate a regulatory effect. For clarity, these are not indicated on this diagram.

### 1.5.6 The latent lifecycle

HSV1 enters sensory neurons innervating the cells of the mucosal membranes. It is then transported to the neuronal cell body where it is able to persist in an episomal state for the lifetime of the host. Periodic reactivation of HSV1 can occur, leading to lytic infections in the same dermatomal distribution as the initial infection. Reactivation can occur spontaneously or can be induced by stress, either directly to the neuron or to the whole organism.

Analysis of viral gene expression during latency has revealed that no genes of the lytic cycle are expressed. Transcription is limited to a single region within the long repeats of the HSV1 genome, giving rise to the production of the latency associated transcripts (LATs) (Stevens *et al.*, 1987). The genetic organisation of the LAT region is shown in figure 1.5.6.1. The major LATs are 2kb and 1.5kb non-polyadenylated RNA species that remain intranuclear. These appear to be stable introns spliced from an unstable 8.3kb primary LAT which is transcribed antisense and complementary to the coding sequence for ICP0. The promoters which control the expression of the LATs are named latency associated promoters 1 and 2 (LAP1 and LAP 2). LAP1 is a TATA-box containing promoter located 700-1300bp upstream of the primary 8.3kb LAT. LAP1 contains basal promoter elements, including a TATA box, ATF/CREB and USF-1 sites (Soares *et al.*, 1996; Bloom *et al.*, 1997). LAP1 is active in most cell types but contains an enhancer element giving it increased activity in neurons (Zwaagstra *et al.*, 1990; Batchelor and O'Hare, 1990). LAP2 is a TATA-less promoter located downstream of LAP1, immediately 5' of the stable 2kb LAT intron (Goins *et al.*, 1994). LAP2 contains several potential *cis* acting elements which have been proposed to increase the promoter activity of LAP2 in the absence of a TATA box. These include an Sp1 site and putative AP2 and E2F sites (Goins *et al.*, 1994). LAP2 also contains CT rich and polyT elements which bind certain transacting factors known to regulate cellular housekeeping gene promoters. Construction of a series of LAP1 and LAP2 deletion mutants has defined the contribution made by each promoter to LAT expression during lytic and latent infection. It was determined that the expression of the 2kb LAT depended almost exclusively on LAP2 during the lytic cycle but on LAP1 during latency (Dobson *et al.*, 1989; Nicosia *et al.*, 1993; Chen *et al.*, 1995).



**Figure 1.5.6.1 The genetic organisation of the LAT region**

The LAT region is located in the long repeat regions of the genome and is therefore present in two copies as indicated. The primary LAT is an unstable 8.3kb transcript. The major species are the 2kb and 1.5kb LATs, which may be smaller spliced introns from the primary LAT. It can be seen from the diagram that the LATs are transcribed antisense and at least partially complementary to the gene encoding ICP0. The two latency associated promoters, LAP1 and LAP2 are indicated.

Understanding the function of the LATs and the mechanisms which control the establishment, maintenance and reactivation from latency remains one of the most controversial and challenging subjects in the field.

Firstly, it is not clear at what level the block to virus replication occurs. The inherent toxicity of all the regulatory IE gene products (Johnson *et al.*, 1994) and the observation that no IE gene mRNA can be detected from latently infected cells has led to the suggestion that the block must be at or before the IE gene level. It was shown that a virus with a mutation which prevents the interaction of VP16 with Oct-1 and HCF (the *in1814* mutation, Ace *et al.*, 1989) was able to establish latency at least as efficiently as wild-type virus (Steiner *et al.*, 1990; Valyi-Nagy *et al.*, 1991). This observation lends further support to the hypothesis that the block to replication during latency occurred before the level of IE gene transcription. The search for factors which were different between cells which established lytic and latent infections began and as a result a variety of explanations for the block to IE gene transcription have been proposed.

One possibility is based on the suggestion that in cells which are not permissive for a lytic infection, splice variants of the POU domain protein Oct-2 (Oct-2.4 and 2.5) serve to compete with Oct-1 for the TAATGARAT binding site (Lillicrop *et al.*, 1991). These Oct-2 isoforms cannot interact with VP16 and so transactivation of the IE gene promoters does not ensue (Lillicrop *et al.*, 1993). The authors reported that when these Oct-2 splice variants were transfected into cells which were permissive for HSV1 replication, IE gene expression was dramatically reduced. It was proposed that this would then abort the lytic infection at an early stage and force the virus to enter latency. This theory therefore suggests that it is the presence or absence of certain transcription factors which determines whether a lytic or latent infection is established. In support of this, Oct-1 is a ubiquitous cellular protein whereas Oct-2.4 and 2.5 are only found in neurons, consistent with the primary sites of establishment of lytic and latent infections, respectively. However, a number of attempts to reproduce these findings have uncovered some inconsistencies and it appears possible that Oct-2 is only found in a subset of the neurons which are capable of harbouring a latent HSV1 infection

(Hagmann *et al.*, 1995; Turner *et al.*, 1996). This suggests that if Oct-2 is involved in the repression of IE gene transcription, it may not be the only factor.

Other possibilities for the block to IE gene transcription include the observation that HCF has different subcellular distributions in neuronal and non-neuronal cells. In most cell types, the distribution of HCF is nuclear (Kristie *et al.*, 1995; La Boissiere *et al.*, 1999) but in neurons it is exclusively cytoplasmic (Kristie *et al.*, 1999). Advocates of this theory would suggest that the lack of nuclear HCF prevents transactivation of IE gene promoters in neurons, forcing the virus into a state of latency. However, experiments which have shown that latency can be efficiently established even in the presence of functional VP16 have suggested that the answer may not be this simple (Sears *et al.*, 1991).

Other proposed contributors to the repression of IE gene transcription are two proteins encoded by RNA transcribed divergently from ICP0 mRNA. These two proteins are transcribed from two open reading frames called ORF P and ORF O and their functions remain unknown (Lagunoff and Roizman, 1994; Lagunoff and Roizman, 1995; Randall *et al.*, 1997). The proposal that they play a role in the establishment of latency is based on the observation that the RNA encoding them overlaps, in the sense orientation, the 8.3kb primary LAT transcript. One proposed function of these proteins is based on the observation that the ORF P protein binds to splicing factors and therefore might inhibit the production of the spliced mRNAs encoding ICP0 and ICP22 which are important in the early stages of infection (Bruni and Roizman, 1996). However, acceptance of this hypothesis requires evidence that ORF P and ORF O are expressed during latent infections and an explanation of the observation that latency can be efficiently established in the absence of this region of the genome (Lee and Schaffer, 1998).

Since the LATs are the only gene products transcribed during latency, it would seem reasonable to assume that they must have some role in either the establishment, maintenance or reactivation from latency. However, what this role is has remained elusive and there are many conflicting reports in the area. Several possibilities for the functions of the LATs will be briefly discussed below.

The fact that the LATs are transcribed antisense and complementary to ICP0 and the observation that ICP0 is a powerful transactivator of IE genes has led to the suggestion that the LATs themselves function to repress IE gene expression by preventing or reducing the expression of ICP0. This view was supported by the observations that the 2kb LAT could inhibit the transactivation of the HSV1 TK promoter by ICP0 (Farrell *et al.*, 1991), that mutant viruses unable to make the 2kb LAT led to increased accumulation of ICP0 RNA (Arthur *et al.*, 1998) and that the LATs can <sup>suppress</sup> ~~supers~~ IE gene expression in a neuronal cell line (Mador *et al.*, 1998). This theory would therefore predict that a virus which was deleted for both copies of LAT would lose the ability to repress ICP0 expression and therefore reactivate from latency with enhanced efficiency. In fact, the opposite appears to be true with numerous reports demonstrating that LAT negative mutants are impaired for reactivation *in vivo* (Leib *et al.*, 1989; Hill *et al.*, 1990; Perng *et al.*, 1994; Bloom *et al.*, 1996). However, whether this impaired reactivation is simply a downstream effect of a reduced efficiency in latency establishment is a debatable point (Thompson and Sawtell, 1997). Working with the hypothesis that LAT negative mutants do exhibit an impaired reactivation from latency, Wechsler and colleagues have tried to identify the region responsible for this phenotype. They showed that the first 1.5kb of the primary 8.3kb LAT was entirely sufficient for wild type levels of reactivation and that most of this reactivation function was contained within the first 811 nucleotides, at least in a particular rabbit model of HSV1 latency (Perng *et al.*, 1996; Drolet *et al.*, 1999). The regions which they implicated as being important for reactivation do not overlap with the transcript for ICP0, leading this group to dismiss the antisense regulation of ICP0 hypothesis.

The observation of several ORFs within the LAT region has led to the suggestion that LAT might encode a protein which functions in the establishment, maintenance or reactivation from latency. This seems a likely hypothesis as it is well known that most gene phenotypes are mediated via a protein. Furthermore, the LAT region of a related  $\alpha$ -herpesvirus, bovine herpes virus (BHV1) has been found to encode a protein which possibly functions to prevent the death of latently infected neurons (Hossain *et al.*, 1995; Schang *et al.*, 1996). However, the observation that no detectable phenotype was observed when the HSV1 LAT ORFs were mutated and the fact that the

LAT transcripts are intranuclear species has led to a general lack of support for this theory. Furthermore, it has been reported that the first 1.5kb of the primary LAT (the region proposed to be important in reactivation) does not contain any ORFs which are well-conserved between three different strains of HSV1, suggesting that the likelihood of this region encoding a functional protein was small (Drolet *et al.*, 1998). However, conserved ORFs have been identified downstream of this 1.5kb region in the major 2kb LAT. The deregulated expression of the largest of these highly conserved ORFs has been shown to significantly enhance the growth of both wild type viruses and viruses deficient in IE gene synthesis, most notably a virus mutated for ICP0 (Thomas *et al.*, 1999b). This led these authors to propose a model whereby the LAT RNA acts as an antisense transcript to ICP0 during the establishment and maintenance of latency but expresses a protein during reactivation, compensating for the lack of ICP0 and other IE gene expression at this time and facilitating the re-initiation of the lytic cycle. The proposed tight regulation of the expression of this protein could account for the lack of previous detection of the protein, despite numerous attempts. Since the transcripts would only be translated relatively rarely, this model also accounts for the fact that the usual localisation of the LATs is nuclear, a fact which has previously been used to discredit the likelihood of protein expression. However, it is harder to reconcile this model with the observed lack of phenotype when the LAT ORFs have been mutated and the finding that the reactivation function apparently lies entirely within the first 1.5kb of the primary LAT.

It is possible that LAT functions directly as RNA. This could be as catalytic RNA or, more likely, as an RNA transactivator. Whilst there is no evidence to suggest that HSV1 LAT might function in this way, a direct RNA-mediated transactivation of transcription from a viral RNA has been reported to occur in red clover necrotic mosaic virus (Sit *et al.*, 1998). Here, a 34-nucleotide stretch of RNA was found to be necessary and sufficient to transactivate the promoter of the gene encoding the viral capsid protein. The report by Thomas and colleagues discussed in the preceding paragraph discounted the possibility that LAT could be acting at the RNA level by including a frameshift mutation in the LAT ORF under study (Thomas *et al.*, 1999b). The inclusion of this mutation removed the growth advantages conferred by LAT, leading the authors

to conclude that LAT was acting as a protein. Northern blots precluded the possibility that this mutation had destabilised the RNA but the possibility that it had altered the function of the RNA was not discussed.

The last possible function of the LATs which will be considered here is that they simply function physically to maintain the genome in a transcriptionally active form such that it is poised to reactivate in response to appropriate stimuli by an undetermined mechanism. This theory would predict that the fact that the LAT transcripts are antisense to ICP0 and that they contain ORFs are both merely coincidental. The most likely situation is that the LAT region has multiple functions, probably including each of the above.

## **1.6 HSV1 based vectors**

HSV1 vectors can be divided into two types, defective vectors or disabled vectors. The relative merits of each type will be briefly discussed below.

### **1.6.1 Defective HSV1 vectors (amplicons)**

Amplicons are defective HSV1 based viruses, incapable of replicating in the absence of helper functions. Amplicon vectors are created from plasmids which contain the transgene, an HSV1 packaging signal (the 'a' sequence, see figure 1.5.1.1) and an HSV1 origin of replication (Spaete and Frenkel, 1982). Traditionally, the plasmid is co-transfected with an HSV1 helper virus into a cell line which supports the growth of the helper virus. The plasmid can replicate in concatamers of up to a viral genome length (152kb) using the replication machinery from the helper virus acting on its HSV1 origin of replication. These concatamers are then packaged into capsids by virtue of their 'a' sequences. On subsequent harvesting, a mixture of defective and helper virus is obtained and there is no way of physically separating the two populations. A schematic diagram of the amplicon system is shown in figure 1.6.1.1. Helper viruses which are traditionally used contain temperature sensitive (ts) mutations or deletions in the essential IE gene ICP4. The advantages of the amplicon system are that the only manipulation required is that of the amplicon plasmid and each replication defective



vector contains multiple copies of the gene of interest. Furthermore, the efficiency of gene delivery is high and the toxicity of the vector is low.

Studies using an amplicon vector expressing  $\beta$ -galactosidase under the control of the ICP22/47 promoter (pHSVlac) have demonstrated high level stable expression of the transgene in cultured neurons of the PNS (Geller and Breakefield, 1988) and CNS (Geller and Freese, 1990) with no reported evidence of cytopathic effect.

In contrast to the disabled HSV1 vectors which will be described in the following section, the choice of promoter driving the expression of the transgene in amplicon vectors is not crucial. This is because the lack of HSV1 DNA means that amplicons are not subject to the same control mechanisms which shut down gene expression at the onset of latency in disabled vectors. Expression from an amplicon can therefore be expected to be maintained for as long as the DNA remains undegraded within the cell. This flexibility in promoter choice has enabled long term, cell-type specific expression to be obtained from amplicons, something which has not yet been achieved with the disabled HSV1 vectors. This was demonstrated by an amplicon study which delivered *lacZ* under the control of the preproenkephalin promoter to the rat brain *in vivo*. In this study it was found that the pattern of transgene expression followed the pattern of endogenous preproenkephalin and was detectable for two months post injection (Kaplitt *et al.*, 1994a).

The amplicon technology has also been successfully used to deliver a number of physiologically active genes both *in vitro* and *in vivo*. These include the delivery of nerve growth factor (NGF) to the rat superior cervical ganglion *in vivo* which was shown to facilitate the maintenance of TH levels after axotomy (Federoff *et al.*, 1992). In culture, expression of the p75<sup>hNGFR</sup> NGF receptor in fibroblasts has produced functional high affinity binding of NGF (Battleman *et al.*, 1993). Other studies have demonstrated that delivery of the glucose transporter gene to the rat hippocampus *in vivo* can reduce CA3 neuron loss following kainic acid delivery (Ho *et al.*, 1993; Lawrence *et al.*, 1996), and delivery of growth associated protein 43 (GAP43) to non neuronal cells produces neurite-like processes (Verhaagen *et al.*, 1994).

Paradoxically, probably the most successful amplicon based study also demonstrated the major limitation of the technology. The study conducted by During *et*

*al.* in 1994 used this system to deliver the gene for TH into the striatum of 6-hydroxydopamine unilaterally lesioned rats. Behavioural recovery in these Parkinsonian rats was assessed by reduction in apomorphine induced rotation rate and significant behavioural and biochemical recovery was maintained for 1 year after gene transfer (During *et al.*, 1994). However, 10% of the rats in this study died within two weeks of gene transfer, and this was proposed to be due to contamination of the amplicon stocks with helper virus which had reverted to wild type.

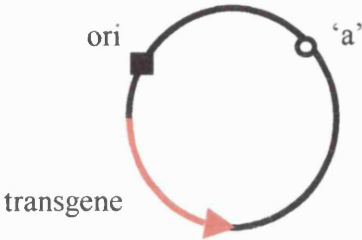
A number of attempts have been made to increase the amplicon to helper virus ratio. These have included conferring a selection advantage on the amplicon (Zhang *et al.*, 1998a), or making the propagation of the two populations mutually dependent on each other (Pechan *et al.*, 1996). More elegantly, the system has been improved by using a set of cosmids that overlap and represent the genome of HSV-1 but are deleted for the DNA cleavage/packaging signals (Fraefel *et al.*, 1996). Due to this deletion, any HSV-1 genomes that are reconstituted from the cosmids via homologous recombination are not packageable. This technique enables the production of 'helper-free' amplicon stocks. An extension of this system has led to the expression of the HSV-1 genome (minus packaging signals) as a bacterial artificial chromosome (BAC) in *E. coli* (Saeki *et al.*, 1998). It is this 'helper free' amplicon system which is shown in figure 1.6.1.1.

Despite these significant improvements in the amplicon technology, several potential limitations remain. Firstly, while amplicon DNA can be maintained in the nucleus for extended periods of time, it will eventually be degraded by cellular enzymes. The technology does not take advantage of the ability of HSV1 to establish a latent infection. However, the inclusion of the Epstein-Barr virus (EBV) unique latent replication origin (ori P) sequence and latency associated nuclear antigen gene (EBNA-1) in amplicons might allow the vector to be maintained as a replicative episome in transduced cells (Wang *et al.*, 1997), although the relevance of this advance to neuronal gene delivery is limited. Secondly, on a more practical note, the fact that several plasmids have to be cotransfected means that the system is somewhat cumbersome and not readily amenable to scale-up.

#### **Figure 1.6.1.1 The HSV1 amplicon system**

A plasmid containing the transgene and *cis*-acting sequences responsible for replication and packaging (ori and 'a' respectively) is cotransfected into permissive cells with a HSV1 cosmid set which provides essential viral proteins in *trans*. The amplicon DNA is then replicated and packaged into HSV virions, producing a pure stock of defective virus containing the transgene. The fact that the cosmid set is deleted for the packaging sequences means that any resulting wild type genomes cannot be packaged. The functions supplied by the cosmid set were traditionally provided by a helper virus, generating a mixed population of defective viral vector and helper virus.

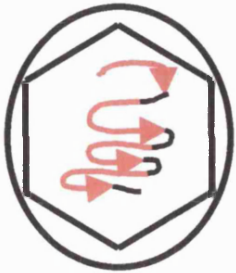
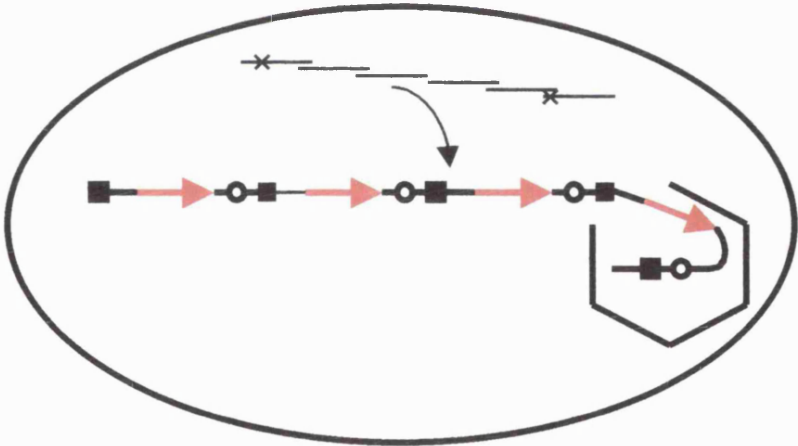
amplicon plasmid



HSV1 cosmid set  
(pac sequences deleted)



cotransfection



Defective HSV1 vector  
containing transgene

### 1.6.2 Disabled HSV1 vectors

The second method commonly used for the generation of HSV1 based vectors is to recombine the transgene and its regulatory sequences directly into the HSV1 genome by cotransfection of plasmid and infectious viral DNA into complementing cells (reviewed in Fink *et al.*, 1996). The recombination site is dictated by homologous HSV1 sequences in the plasmid 5' and 3' of the transgene and its regulatory sequences. Essential IE genes can be 'knocked out', rendering all the recombinant progeny replication deficient. Selection and plaque purification of the recombinant progeny is aided by the use of reporter genes such as the bacterial protein *lacZ* or the jellyfish green fluorescent protein (GFP). For the subsequent construction of viruses containing genes of interest, this reporter gene cassette can then be 'knocked out' and colourless 'white' plaques selected and purified away from the coloured background. The cotransfection, purification and subsequent growth of the vector are carried out on a cell line which complements the deleted essential gene(s). In this way, a pure stock of the disabled vector containing the gene of interest can be obtained. Disabled HSV1 vectors are easy to quantitate by titration and plaque assay. As each viral genome contains a known copy number of the transgene (either one or two depending whether the insertion site was in the unique or repeated regions of the genome), the dose of the transgene can be controlled and easily reproduced. This is in contrast to amplicons where individual vectors may contain differing numbers of concatemers of the transgene, potentially making experiments less reproducible.

There have been two main limitations which have hindered the use of disabled HSV1 as a vector for gene delivery to neurons. Firstly, as HSV1 does not integrate into the host genome, and the majority of HSV1 and heterologous promoters are switched off at the onset of latency, expression of transgenes has mainly been transitory. Secondly, the wild type virus is highly pathogenic; cerebral injection causes a fatal encephalitis. The virus must therefore be disabled prior to its use as a gene delivery vector. However, even in a replication incompetent virus, expression of HSV1 proteins can still be highly cytotoxic. Advances towards overcoming these two limitations are discussed below.

### 1.6.2.1 Long term expression of the transgene

Early studies on disabled HSV1 vectors utilised promoters of genes expressed during the lytic cycle to drive the expression of a transgene from a lytic gene locus. Transgenes were placed under the control of the *tk* promoter, the ICP0 promoter, the ICP4 promoter, the ICP6 promoter, the gC promoter or the ICP8 promoter (Ho and Mocarski, 1988; Palella *et al.*, 1988; Chiocca *et al.*, 1990). The transgene cassettes were inserted into lytic gene loci and the viruses injected into the CNS or PNS of mice. In all these cases, high expression levels of the transgene were detected for up to 4 days post infection, i.e. during the acute stage of the infection but expression was rapidly shut off as the virus entered latency. This shut off of transcription was also found to apply to heterologous, non-HSV1 promoters. These included the IE human cytomegalovirus (CMV) promoter in the US3 locus (Fink *et al.*, 1992) and the metallothionein, phosphoglycerate kinase or Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoters in the gC locus (Lokensgard *et al.*, 1994). However, despite its inactivity during latent phase transcription when in the gC locus, the MMLV LTR promoter was shown to facilitate long term gene expression when inserted into the ICP4 locus (Dobson *et al.*, 1990). In this study, expression of *lacZ* was observed for at least 24 weeks in the PNS and up to 5 weeks in the CNS. This observation was somewhat surprising at the time although with hindsight it can be explained by the fact that the orientation of the insertion (antisense to ICP4) placed the MMLV LTR promoter 3' of and in the same orientation as the LAT promoters. The unusual characteristics of the MMLV LTR promoter will be discussed further later.

Exploiting the long term transcriptional activity of the LAT promoters is an obvious means by which continuing expression of a transgene might be achieved. As discussed in section 1.5.6, expression of the LATs is controlled by two promoters, LAP1 and LAP2. Early attempts to take advantage of the LAT promoters to drive expression of exogenous genes concentrated on the core LAT promoter, LAP1. The existence of LAP2 was unknown at this time but it became apparent that the potential for long term expression from LAP1 depended on the exact insertion site of the transgene within the LAT region. When rabbit  $\beta$ -globin RNA or the *lacZ* or NGF genes were placed immediately downstream of the LAP1 TATA box, expression was detected

for 3 weeks, but at a reduced level to that initially achieved (Dobson *et al.*, 1989; Margolis *et al.*, 1993). However, when the insertion site was further downstream from the LAP1 TATA box (with hindsight, after LAP2) expression from a *lacZ* transgene could be detected in mouse trigeminal ganglia for 8 weeks post injection (Ho and Mocarski, 1989). The authors reported that the expression levels were low, that the staining was punctate *in vivo* and that expression was undetectable in vero cells in culture, all observations now known to be characteristic of LAP2-driven expression. Expression of the  $\beta$ -glucuronidase gene under the control of LAP1 and what has now been identified as the 5' end of LAP2 was maintained for four months in the trigeminal ganglia and brainstems of mice (Wolfe *et al.*, 1992). A number of other studies served to confirm that LAP1 on its own was insufficient to allow continued expression during latency. These studies included the insertion of a LAP1-*lacZ* cassette into the gC locus, a site far removed from the LAT region (Dobson *et al.*, 1995). In this study it was reported that expression of the transgene decreased over several weeks, presumably corresponding to the onset of latency.

It therefore appeared that elements downstream of LAP1 were necessary to facilitate the long term expression of transgenes from HSV1 vectors. The identification of LAP2 in this downstream region led to a number of studies aimed at determining whether this promoter was necessary and sufficient for long term transgene expression. Although the data was not shown, the report that a LAP2-*lacZ* cassette inserted into the gC locus could express the transgene in mouse dorsal root ganglia for at least 300 days further suggested that the determinant of long term expression had been found (Goins *et al.*, 1994). However, the expression afforded by LAP2 in this ectopic site was said to be very weak. In fact, when this virus was used in direct injections into the hippocampus, histochemical staining with X-Gal could not detect any  $\beta$ -galactosidase expression but *lacZ* mRNA was apparently detectable by RT-PCR 4 weeks post injection (Fink *et al.*, 1996). The benefits of the higher level, neuron specific expression obtainable from LAP1 and the long term activity offered by LAP2 have been combined in a virus which uses an internal ribosome entry site (IRES) from encephalomyocarditis virus (Lachmann and Efstathiou, 1997). These authors inserted the IRES after the LAP2 region, allowing the transgene to be transcribed from LAP1 but without disrupting the

structural integrity of the LAT region. This virus was found to express *lacZ* for at least 190 days in the PNS and at least 307 days in specific regions of the CNS. Importantly, unlike when expression is driven from just LAP1, the number and intensity of staining of *lacZ* positive cells detected in this study increased with the onset of latency, suggesting that true latent phase expression had been obtained.

It has also been found that the LAP2 region is able to confer long term expression on LAP1 at ectopic sites in the genome. The LAP2 region downstream of LAP1 in the gC locus was able to drive long term expression in the PNS for at least 28 days (Lokensgard *et al.*, 1997). This study also explored the versatility of the LAP2 region by the construction of a number of recombinant viruses which contain this region juxtaposed to LAP1 in a number of different ways. It was reported that although the LAP2 region was able to confer long term expression when it was upstream of and in the reverse direction to LAP1, the element functioned best when it was in its natural orientation and position downstream of LAP1. In a later publication, these authors developed a more sensitive assay and speculated that this apparent directionality might be attributable to lytic phase promoter activity from the LAP2 region. It was suggested that promoter activity from the LAP2 region during the lytic stages of infection might raise the initial levels of transgene expression levels so high that there was more “residual” expression at the later time points. When the LAP2 region was in the reverse orientation, this lytic stage promoter activity would not effect transgene expression, resulting in both lower initial and residual expression levels (Berthomme *et al.*, 2000). This hypothesis then raises the question of whether the LAP2 region does actually confer long term expression capabilities onto a neighbouring promoter or whether the enhancer function of this region is sufficiently strong to make this appear to be the case. However, long term expression studies with LAP2 alone (Goins *et al.*, 1994) or with LAP1/LAP2 in a replication incompetent virus (Marshall *et al.*, 2000) would support the idea that the enhancer and long term expression capabilities of the LAP2 region are two independent functions, the former but not the latter having a directionality when in the context of a viral genome.

The unusual characteristics of the MMLV LTR promoter were mentioned previously. The early work utilising this promoter in HSV1 vectors demonstrated that it



was capable of driving long term expression of an exogenous gene when inserted into the ICP4 locus (Dobson *et al.*, 1990). The observation that other promoters were not active during latency in this locus (Chiocca *et al.*, 1990) and that the MMLV LTR promoter was not active during latency in other insertion sites (Lokensgard *et al.*, 1994) suggested that something peculiar to the MMLV LTR promoter allowed it to function long term when inserted in a site close to the endogenous LAT region. This observation was investigated further by Lokensgard and colleagues who constructed a LAP1/MMLV LTR hybrid promoter (Lokensgard *et al.*, 1994). This promoter consisted of a TATA-less LAP1 fused to a MMLV LTR-*lacZ* cassette inserted into the ectopic locus of gC. It was found that this hybrid promoter was capable of directing high level, long term expression of the transgene for at least 42 days post injection in the PNS. Similar constructs containing LAP1 in combination with a number of other promoters did not have this effect, indicating that the finding was specifically related to the MMLV LTR promoter. It has also been reported that an MMLV LTR promoter inserted directly upstream of and in the opposite direction to LAP1 within the endogenous LAT region can drive transgene expression for at least 18 months post infection (Carpenter and Stevens, 1996). However, in this study expression was only initially detected in a very small number of cells and significantly decreased over time. Thus it appears that the MMLV LTR promoter may have characteristics which allow it to functionally substitute for LAP2 in conferring long term expression capabilities on LAP1.

From the foregoing discussion, it is apparent that LAP1 alone is insufficient at driving the long term expression of an exogenous gene. However, LAP2, either on its own or in combination with LAP1 is capable of directing the stable expression of transgenes through latency. It also appears that the function that is provided by LAP2 in conferring this long term expression on LAP1 can be substituted for by the MMLV LTR promoter. This might suggest that the function of the LAP2 and MMLV LTR promoters is primarily a structural one, scaffolding the genome to allow continued access of transcription factors to LAP1. This hypothesis will be discussed further in chapter 3.

### 1.6.2.2 Reducing cytotoxicity

For unknown reasons which were discussed in section 1.5.6, HSV1 rarely enters a lytic replication cycle in neurons during natural infections. However, following stereotaxic injection directly into the CNS, or infections of primary cultures at high multiplicities, lytic replication does occur. Such lytic replication rapidly destroys cells in culture or causes encephalitis and death of a test animal. In contrast, latent infections of neurons do not cause any detectable detrimental effects to the physiology of the cell. It is therefore clear that wild type HSV1 must be disabled in some way such that lytic replication is blocked and the virus is forced to enter latency. These disablements can be broadly divided into two classes, deletion of essential genes or deletion of non-essential genes. Non-essential genes provide accessory functions which contribute to pathogenicity *in vivo* but are dispensable for growth in cell culture whereas essential genes are absolutely required both for growth in culture and for pathogenicity.

Non-essential genes often encode functions which are only required in certain cell types, such as neurons. For example, the genes encoding thymidine kinase (*tk*), the large subunit of ribonucleotide reductase (ICP6) and ICP34.5 are only essential for growth in non-dividing cells (Cameron *et al.*, 1988; Chou *et al.*, 1990; MacLean *et al.*, 1991). These 'neurovirulence factors' can therefore be deleted without adversely affecting growth in culture but rendering the virus unable to replicate in neurons. Vectors have been constructed with deletions in one or more of these neurovirulence factors. Early viruses contained deletions only in the *tk* gene (Ho and Mocarski, 1988; Palella *et al.*, 1988). Although these mutants did show reduced virulence in neurons, they were still lethal after high titre inoculation, presumably due to replication in non-neuronal cells (Palella *et al.*, 1989). Disablement of ICP34.5 is reported to provide the greatest degree of neuroattenuation of any of the non-essential genes, its deletion increasing the LD<sub>50</sub> by a factor of 10<sup>6</sup> over wild type virus following intracranial inoculation in mice (Chou *et al.*, 1990). However, viruses deleted for only ICP34.5 were found to afford very low efficiency of transduction of neurons in both the PNS and CNS (Coffin *et al.*, 1996). Furthermore these viruses expressed viral antigens which elicited a host immune response and caused severe inflammation in the brain (McMenamin *et al.*, 1998a; McMenamin *et al.*, 1998b). Whilst these neuroattenuated viruses may not be

suitably disabled for the majority of applications, their ability to divide in replicating but not non-replicating cells has suggested that they might be appropriate for the treatment of gliomas. Indeed, viruses deleted for either just ICP34.5 or ICP34.5 in combination with ICP6 are currently being used to treat end-stage glioma patients in clinical trials (Mineta *et al.*, 1995; Markert *et al.*, 2000; Rampling *et al.*, 2000).

It therefore became apparent that whilst viruses deleted for these non-essential genes can be grown to high titres in culture and do not require the use of a complementing cell line, the resulting vectors are still replication competent and can enter the lytic cycle if used at high MOIs. This suggested that a combination of essential and non-essential genes would need to be deleted if a non-cytotoxic virus was to be generated.

The cascade nature of HSV1 gene expression suggested that the IE genes were a sensible target for disablement. Early vectors contained deletions or insertions in the essential IE gene encoding ICP4 generating viruses which were incapable of undergoing a productive infection, except in a complementing cell line which provided ICP4 in *trans* (DeLuca *et al.*, 1985; Chiocca *et al.*, 1990; Dobson *et al.*, 1990; Johnson *et al.*, 1992). The deletion of ICP4 caused a dramatic reduction in HSV1 gene expression, with the vast majority of the 80 or so HSV1 genes now not being expressed. However, the remaining IE genes ICP0, ICP22, ICP27 and ICP47 and the hybrid IE/E gene ICP6 were still found to be expressed from this virus (DeLuca *et al.*, 1985). Stereotaxic injection of these ICP4 deleted viruses into the CNS demonstrated that they were of considerably reduced virulence compared to the wild type virus, with 3 out of 3 animals surviving 14 days post injection as compared to 1 out of 8 of the animals injected with wild type virus (Chiocca *et al.*, 1990). However, it was noted that there was considerably more tissue damage in the CNS of animals injected with the ICP4 deleted virus compared to animals injected with media alone.

Further evidence for the toxicity of the ICP4 deleted viruses came from the observation that fibroblasts infected with these viruses exhibited chromosomal abnormalities, fragmentation of cellular DNA and cytoplasmic blebbing (Johnson *et al.*, 1992). Likewise, individual deletions in ICP0 or ICP27 or the combined deletion of ICP4/ICP22 or ICP4/ICP47 generated viruses which were still toxic to a number of cell

types, including neurons (Johnson *et al.*, 1992). Indeed, the individual deletion of ICP0 produced a virus which was significantly more toxic *in vivo* than one just deleted for ICP4, presumably due to the fact that deletion of only this non-essential IE gene left the virus with a degree of replication competency (Chiocca *et al.*, 1990). The advantages of combining deletions in essential and non-essential genes were demonstrated by a report which found that a virus deleted for ICP34.5 and ICP27 was not only less toxic but also afforded significantly higher levels of transgene expression than a virus deleted for ICP34.5 alone (Howard *et al.*, 1998).

The possibility that the virion itself was toxic to cells was excluded by using UV-irradiated viral stocks or cells pre-treated with interferon, both of which would considerably reduce or abrogate viral gene expression. These studies demonstrated that the observed cytotoxicity was attributable to a factor or factors being expressed from the virus genome (Johnson *et al.*, 1992). A later study by the same group went on to demonstrate that the products of the IE genes ICP0, ICP27 and ICP22 were themselves toxic in transient transfection assays, as determined by their ability to impair transformation of fibroblasts when cotransfected with a plasmid encoding neomycin resistance. It was therefore concluded that the expression of ICP0, ICP27 and ICP22 was likely to contribute to the toxicity of an ICP4 deleted virus whereas the products of the ICP47 and ICP6 genes were less likely to contribute (Johnson *et al.*, 1994).

This hypothesis was supported by the observation that viruses with deletions in the IE genes ICP4, ICP22 and ICP27 were significantly less toxic than the single or double mutants (Wu *et al.*, 1996). It was reported that this triple deletion virus allowed cells infected at a multiplicity of infection (MOI) of 10 to be maintained in culture for at least three days post infection. This was in contrast to any of the doubly disabled viruses which only allowed cells infected at this high MOI to be maintained for one day post infection. These differences were further quantified by an analysis of cellular  $\beta$ -tubulin. This analysis revealed that there was significantly less cellular  $\beta$ -tubulin RNA 6 and 24 hours post infection with viruses deleted for ICP4 only or ICP4 and ICP27 than in mock infected cells or cells infected with viruses deleted for ICP4, ICP27 and ICP22. The authors concluded that the stability or the transcription of cellular transcripts was compromised in cells infected with the less disabled viruses (Wu *et al.*, 1996).

A virus deficient in ICP4, ICP27 and ICP22 has also been constructed by another laboratory (Krisky *et al.*, 1998b). These authors also report a considerable reduction in cytotoxicity with this triple deletion virus compared to viruses deleted for ICP4 and ICP27 or ICP4 and ICP22. Furthermore, this paper demonstrated that primary cortical or dorsal root ganglia neurons infected at low MOI with the triple mutant virus survived for over 21 days in culture without obvious morphological change or cell death. This was in marked contrast to a virus deleted for ICP4 only which had killed all the neurons by 4 days post infection. However, despite these promising observations, it was found that vero cells infected with a virus deleted for ICP4, ICP27 and ICP22 are inhibited for DNA synthesis and cell division (Wu *et al.*, 1996). In addition, the appearance of cells infected with the triple deletion mutant differed from uninfected cells in that the former had large nuclear inclusions which were found to contain high levels of the product of the remaining regulatory IE gene, ICP0. These inclusions were not observed in a virus deleted for ICP4 only, or a virus deleted for ICP4 and ICP22, leading the authors to conclude that the inclusions were a result of the deletion of ICP27. This conclusion is in keeping with one of the known actions of ICP27 in the context of an ICP4 deleted virus, which is to cause ICP0 to be localised predominantly in the cytoplasm (Zhu *et al.*, 1994; Zhu *et al.*, 1996).

The preceding studies suggested that the expression of all the regulatory IE genes would need to be prevented in order to generate a virus which was completely non-cytotoxic. Such a virus was subsequently generated (Samaniego *et al.*, 1998). As might be expected from the cascade nature of HSV1 gene expression, cells infected with this virus showed polypeptide expression profiles indistinguishable from that of uninfected cells, indicating that little or no HSV1 protein is made by this virus. As a result of this, the virus was completely non-toxic to vero and human embryonic lung (HEL) cells in culture, even 28 days post infection at an MOI of 20. Furthermore, the viral genomes were able to persist in a functional form, demonstrated by the fact that transgene expression from the virally infected cells could be reactivated at any time up to the end of the experiment.

The IE genes ICP4 and ICP27 are essential for replication and must be complemented in culture to enable propagation of the disabled viruses (DeLuca *et al.*,

1985; Sacks *et al.*, 1985). While the remaining IE genes ICP22 and ICP0 are non-essential, their deletion significantly impairs replication, particularly at low MOI (Sears *et al.*, 1985; Chen and Silverstein, 1992; Cai and Schaffer, 1992). In the course of the systematic inactivation of all the regulatory IE genes, it was found that each of them needs to be complemented in culture for efficient propagation of virus stocks. The virus which is deficient in the expression of all the IE genes must therefore be grown on a cell line which provides ICP0, ICP4 and ICP27. Due to the inherent toxicity of all the IE gene products, such cell lines are only suitable for use up to passage 15 and do not offer full and consistent complementation (Samaniego *et al.*, 1997). The mutation which causes the virus to be deficient in ICP22 is a deletion of the promoter region containing the TAATGARAT motif. Since the promoters for ICP22 and ICP47 are identical as they are derived from sequences located within the short repeat regions of the HSV1 genome (Watson *et al.*, 1981), this mutation affects the expression of both genes. The rationale for this deletion was that it would render the promoter unresponsive to VP16 but still responsive to activation by ICP4, on account of the fact that the mutated promoter now resembled that of an early or late gene, promoters known to be activated by ICP4. It was therefore anticipated that the ICP22 (and ICP47) genes would be activated in the presence of ICP4, i.e. in culture, but inactive in the absence of ICP4, i.e. in target cells (Samaniego *et al.*, 1998). In this way, the authors circumvented the need to completely delete the gene encoding ICP22 and then complement the deletion in *trans*.

An alternative approach to the individual deletion or inactivation of all the IE genes (and the resulting necessity to complement them all individually from a cell line), is to disable the gene encoding VP16. As was discussed in section 1.5.5, VP16 is a virion component which enters the nucleus with the viral genome and serves to transactivate the promoters of the IE class of genes. This transactivation is achieved through the formation of a multicomponent complex on the TAATGARAT motifs in the IE gene promoters. VP16 therefore represents an attractive target for disablement as it offers the potential to reduce or prevent the expression of all the IE genes. However, the situation is complicated by the fact that VP16 cannot be deleted from the virus as it also encodes an essential structural component of the virion (Batterson and Roizman,

1983). Therefore, if VP16 was deleted from the virus and provided *in trans* from a complementing cell line (such as is done for the IE genes), the virions would package the cellularly derived VP16 into their tegument and carry it over to target cells, where it would transactivate the IE gene promoters. The beneficial effects of the VP16 deletion would then be lost. This problem was overcome by the identification and subsequent exploitation of the fact that distinct domains in VP16 control the transactivation of IE gene transcription and the assembly of virions (Ace *et al.*, 1988). A 12 base pair linker insertion made into the transactivation domain of VP16 disrupted the interaction of the protein with Oct-1 and HCF and resulted in a virus which was severely impaired in its transactivating function but still able to fulfil its essential structural role (Ace *et al.*, 1989). This virus was named *in1814* and will be referred to a number of times in this thesis. It was reported that this virus was reduced by at least 90% in its ability to stimulate IE gene transcription. However, the virus does not require complementation in culture as it can be grown to high titres by the inclusion of hexamethylbisacetamide (HMBA) in the growth media (McFarlane *et al.*, 1992).

It was found that inclusion of the *in1814* mutation in a virus deleted for ICP4 could significantly reduce the cytotoxicity of the virus, presumably by reducing the expression of ICP0, ICP22 and ICP27 (Johnson *et al.*, 1994). Further work demonstrated that if the *in1814* mutation was combined with conditional mutations in ICP0 and ICP4 a virus which was of reduced cytotoxicity could be generated (Preston *et al.*, 1997). It was reported that this virus caused no visible toxicity to vero cells in culture 24 hours after infection at an MOI of 5. This virus was deficient in all of the major viral transactivators although none had been specifically deleted.

Further developments on a variation of this virus led to the insertion of the IRES-*lacZ* cassette downstream of LAP1 and LAP2, the promoter arrangement which had previously been determined as capable of driving long term expression in a wild type virus (Lachmann and Efstathiou, 1997, discussed in section 1.6.2.1). This generated a conditionally replication incompetent virus which was capable of driving expression of *lacZ* for at least 5 months in the PNS (Marshall *et al.*, 2000). As was the case when the insertion was in a wild type virus, the disabled virus demonstrated increasing transgene expression with time, demonstrating that latent phase gene

expression was occurring. Although this was the first report of authentic latent phase transgene expression from such a disabled virus, it is important to note that this virus does not actually have any genes deleted. The virus is only disabled for one essential gene (ICP4) and the mutation in this gene is temperature sensitive, allowing wild type levels of ICP4 expression at the permissive temperature of 31°C but approximately 10<sup>5</sup> fold less expression at the non-permissive temperature of 38°C (the *tsK* mutation, (Preston, 1979; Watson *et al.*, 1980; Davison *et al.*, 1984). It is therefore possible that some limited replication may have occurred at the site of injection, based on the fact that the footpad of a mouse is likely to be nearer to 31°C than 38°C. The authors addressed this point but suggested that if such replication did occur, the effect of the progeny would be negligible compared to the high titres of virus delivered in the inoculum (Marshall *et al.*, 2000).

### **1.7 Viral vectors in AD research**

Several viral vector systems have been successfully used to deliver AD-related genes to neurons in culture. Indeed, delivery of transgenes to neurons in several “landmark” AD papers has been achieved using the Semliki Forest Virus (SFV) vector system (De Strooper *et al.*, 1998; De Strooper *et al.*, 1999. However, SFV is highly cytotoxic, and this factor severely limited the time course of the experiments in these papers (to less than 24 hours post infection). Due to these problems, SFV is not now a commonly used vector system and was therefore not discussed in the preceding section.

Adenovirus and amplicon HSV1 vectors have also been used to deliver genes relevant to AD to cultured neurons. Adenovirus vectors have been successfully used to determine that overexpression of wild type APP causes neurons in culture to degenerate (Nishimura *et al.*, 1998) and that this degeneration involves activation of caspase 3 and is therefore probably mediated via an apoptotic pathway (Uetsuki *et al.*, 1999). Studies with recombinant adenovirus have also revealed a role for PS1 in the processing and trafficking of a number of neuronal proteins (Naruse *et al.*, 1998; Weihl *et al.*, 1999). HSV1 amplicons have been used in similar experiments to determine the effect that FAD-associated mutations in APP have on the accumulation of  $\beta$ -stubs and A $\beta$  in cultured neurons and astrocytes (McPhie *et al.*, 1997). However, the experiments



reported in this paper were terminated at only 16 hours post infection and the authors reported significant contamination of their recombinant HSV1 stocks with helper virus.

It can therefore be seen that although the use of recombinant viral vectors in AD research has been fruitful, the toxicity of the vector system itself or the contaminating helper virus has limited the scope of some of these experiments. Indeed, one paper which used HSV1 amplicons to study the effects of PS1 on apoptosis reached a conclusion which is contrary to that reported by most others in the field (Bursztajn *et al.*, 1998). This discrepancy has been attributed by some to the toxicity of the viral vector systems employed in this study (Czech *et al.*, 2000). To date, there have been no published reports of delivery of AD-related genes to the CNS *in vivo* using viral vector systems.

## 1.8 Aims of the thesis

Whilst the use of transgenic mouse models of AD have provided much phenomenological data, they cannot provide a reliably predictive model of the human pathology or allow a number of unresolved questions to be addressed. Key amongst these questions is how mutations in either the presenilins or APP, both of which result in an increase in A $\beta$  species in mice and cultured cells, result in the full pathology of AD in humans. This includes A $\beta$  plaques but is also characterised by neurofibrillary tangles composed of hyperphosphorylated forms of the protein tau, a pathology which is not found in rodent models of the disease.

This thesis attempts to address these issues with the development of an HSV1 vector system which can deliver AD-related genes to a variety of rodent and non-rodent neurons both *in vitro* and *in vivo*. HSV1 was used as this virus allows the insertion of multiple genes into a single vector, ensuring that all the exogenous genes are delivered to the same transduced cell. Specifically, the aims of this thesis were as follows:

- to develop a HSV1 based vector system which allowed the high level, long term expression of multiple transgenes.
- to characterise this vector and assess it's ability to deliver transgenes to neurons of rodent and non-rodent origin *in vitro* and *in vivo*.
- to clone human APP and PS1 wild type and mutants into this vector in various combinations and use the system to deliver these genes to neurons in culture. This was aimed at investigating interactions between APP and PS1 and the mechanisms by which mutations in these proteins result in elevated levels of A $\beta$  species. Delivery to neurons from non-rodent origins would potentially provide insights into how the two pathologies of A $\beta$  plaques and tau tangles are linked.
- to use these viruses to deliver APP and PS1 to the CNS *in vivo* with the eventual aim of delivery to the primate brain in an attempt to reproduce both aspects of AD pathology.

## **CHAPTER 2**

# **MATERIALS AND METHODS**

## 2.1 Materials

### 2.1.1 Standard buffers and solutions

The following standard buffers and solutions were used throughout (all concentrations expressed at 1x):

TE: 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0

TAE: 400mM Tris base, 200mM sodium acetate, 20mM EDTA pH 8.3

TBE: 89mM Tris base, 89mM boric acid, 2mM EDTA pH 8.0

PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4mM KH<sub>2</sub>PO<sub>4</sub>

SSC: 150mM NaCl, 15mM sodium citrate

Luria Bertani (LB) media: 1% (w/v) Bacto®-tryptone

1% (w/v) NaCl

0.5% Bacto®-yeast extract

LB media was autoclaved at 120°C for 20 minutes at 10lb/square inch (psi)

Chloroform/Isoamyl alcohol (IAA): 96% (v/v) chloroform, 4% (v/v) IAA

Tris equilibrated phenol: liquefied phenol equilibrated twice with excess 0.1M Tris-HCl pH 8.0

### 2.1.2 Bacterial Strains

**Table II : Description and sources of bacterial strains**

Strain	Genotype	Source
<i>E.coli</i> XL1-blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB</i> <i>LacI</i> <sup>r</sup> <i>ZΔM15</i> , <i>Tn</i> <i>10</i> (Tet) <sup>r</sup> ]	Stratagene Ltd., Cambridge, U.K.
<i>E.coli</i> SCS110	<i>rpsL</i> (Str <sup>r</sup> ) <i>thr leu endA thi-1</i> <i>lacY galK galT ara tonA</i> <i>tsx dam dcm supE44Δ</i> ( <i>lac- proAB</i> ) [F' <i>traD36 proAB</i> <i>lacI</i> <sup>r</sup> <i>ZΔM15</i> ]	Stratagene Ltd., Cambridge, U.K.

### 2.1.3 Cosmids/Plasmids

**Table III: Description and sources of cosmids and plasmids**

Name	Description	Resistance	Source/Reference
pJ4 $\Omega$	Expression vector containing the MMLV LTR promoter and SV40 t intervening sequence (IVS) and polyadenylation signal (pA)	Ampicillin	(Morgenstern and Land, 1990)
pSP72	Cloning vector	Ampicillin	Promega Corporation, Madison, Wisconsin, U.S.A.
pBluescript	Cloning vector	Ampicillin	Stratagene Ltd., Cambridge, U.K.
pcDNA3	Expression vector containing the cytomegalovirus (CMV) immediate early (IE) promoter/enhancer and bovine growth hormone (BGH) pA	Ampicillin	Invitrogen Corporation, Carlsbad California, U.S.A.
pCH110	Eukaryotic assay vector containing the <i>E.coli lacZ</i> gene	Ampicillin	Pharmacia Biotechnology Ltd, St. Albans, Herts, U.K.
pGL2 Basic	Luciferase plasmid	Ampicillin	Promega Corporation, Madison, Wisconsin, U.S.A.
pMAMneo	Mammalian expression vector containing the MMTV LTR promoter and SV40 pA	Neomycin	Clontech Laboratories Inc. Palo Alto, California, U.S.A.
pVgRXR	Plasmid expressing the ecdysone receptor, retinoid X receptor and zeocin resistance genes	Zeocin	Invitrogen Corporation, Carlsbad California, U.S.A.
pSG130BS	ICP27 coding region controlled by ICP27 promoter and pA cloned into pUC18	Ampicillin	(Sekulovich <i>et al.</i> , 1988)
pcDNA/Amp ETIF	EHV1 gene 12 cloned into pcDNA1	Ampicillin	Matt Grapes, Marie Curie Institute, The Chart, Oxted, Surrey

**Table III (continued)**

cosmid human APP 695 WT	Human APP 695 WT cloned into pcos6EMBL	Tetracycline	Karen Hsiao, University of Minnesota, Minnesota, U.S.A. (Hsiao <i>et al.</i> , 1996)
cosmid human APP 695 Swe	Human APP 695 with the Swedish mutation cloned into pcos6EMBL	Tetracycline	Karen Hsiao, University of Minnesota, Minnesota, U.S.A. (Hsiao <i>et al.</i> , 1996)
pBS PS1 WT	pBluescript containing PS1 WT	Ampicillin	Jill Richardson, Neurosciences Unit, Glaxo-Wellcome, U.K.
pBS PS1 A246E	pBluescript containing PS1 with the A246E mutation	Ampicillin	Jill Richardson, Neurosciences Unit, Glaxo-Wellcome, U.K.

#### 2.1.4 Cell lines

**Table IV: Description and sources of cell lines**

Name	Description	Culture Media	Reference/Source
BHK 21 (clone 13)	Baby hamster kidney	1x Dulbecco's modified Eagle's media (DMEM) containing 100U/ml penicillin and 100µg/ml streptomycin and supplemented with 10% (v/v) fetal calf serum (FCS) and 5% (v/v) tryptose phosphate broth (TBP)	Macpherson and Stoker, 1962 ATCC* CCL 10
B130/2	BHK cell line stably transfected with pMAM neo and pSGB130 (ICP27 promoter, coding region and pA)	As for BHK, but with G418 at 800µg/ml to maintain selection	(Howard <i>et al.</i> , 1997)

**Table IV (continued)**

Vero	African green monkey kidney	1x DMEM containing 100U/ml penicillin and 100µg/ml streptomycin and supplemented with 10% FCS	ATCC CCL81
HEK293	Human embryonic kidney	1:1 combination of 1x DMEM and 1x HAMS F12 containing 100U/ml penicillin and 100µg/ml streptomycin and supplemented with 10% (v/v) FCS	(Graham <i>et al.</i> , 1977) ATCC CRL1573
HEK293 APP WT	HEK293 cells stably transfected with APP WT in pcDNA3	As for HEK293, but with G418 at 500µg/ml to maintain selection	Jill Richardson, Neurosciences Unit, Glaxo-Wellcome
HEK293 APP Swe	HEK293 cells stably transfected with APP Swe in pcDNA3	As for HEK293, but with G418 at 500µg/ml to maintain selection	Jill Richardson, Neurosciences Unit, Glaxo-Wellcome

\*ATCC® is the registered trademark of the American Type Culture Collection

## 2.1.5 Antibodies

**Table V: Sources and conditions of use of antibodies**

Antibody	Dilution or Concentration	Secondary	Source
Anti-HSV1 ICP0	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Autogen Bioclear, Caine, Wilts, U.K.
Anti-HSV1 ICP27	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Autogen Bioclear, Caine, Wilts, U.K.
Anti-HSV1 ICP4	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Virusys Corporation, North Berwick, Maine, U.S.A.
Anti-HSV1 VP16	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Matt Grapes, Marie Curie Institute, The Chart, Oxted, Surrey, U.K.

**Table V (continued)**

Anti-HSV1 ICP6	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-rabbit Ig	Barklie Clements, Institute of Virology, Glasgow, U.K.
Anti-HSV1 ICP22	1:500 (Western blot)	1/1000 dilution of HRP-conjugated anti-rabbit Ig	Bernard Roizman, University of Chicago, Illinois
Anti-HSV1 ICP47	1:500 (Western blot)	1/1000 dilution of FITC-conjugated anti-rabbit Ig	David Johnson, Oregon Health Sciences University, Portland, Oregon
Anti-HSV1	1:400 (Immuno fluorescence)	1/500 dilution of FITC-conjugated anti-rabbit Ig	Dako Ltd., High Wycombe, Bucks
Anti-EHV1 gene 12 protein	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Gretchen Caughman, Medical College of Georgia, Georgia, U.S.A.
TUJ1 (anti neuronal specific tubulin)	1:5000 (Immuno fluorescence)	1/100 dilution of rhodamine- conjugated anti- mouse Ig	Babco, Richmond, California
22C11 (anti-N-terminus of human APP)	1:200 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Boehringer Manheim, Lewes, East Sussex, U.K.
Anti-GFP	1:2000 (Western blot)	1/1000 dilution of HRP-conjugated anti-rabbit Ig	Clontech Laboratories Inc. Palo Alto, California, U.S.A.
Tau1 (anti-tau un- phosphorylated at serines 199 and 201)	1:500 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Neuroscience Dept., Institute of Psychiatry, King's College London
TP70 (anti- total tau)	1:10 000 (Western blot)	1/1000 dilution of HRP-conjugated anti-rabbit Ig	Neuroscience Dept., Institute of Psychiatry, King's College London
NT1 (anti-N-terminus of human PS1)	1:1000 (Western blot)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Paul Mathews, Nathan Kline Institute, New York
6E10 (anti-N-terminus A $\beta$ )	1 $\mu$ g/well (ELISA)	1/1000 dilution of HRP-conjugated anti-mouse Ig	Senetek, Missouri, U.S.A.
4G8 (anti A $\beta$ )	1 $\mu$ g/well (ELISA)	1/1000 dilution of HRP-conjugated rabbit anti-mouse Ig	Senetek, Missouri, U.S.A.



**Table V (continued)**

G28 (anti-N-terminus A $\beta$ )	0.5 $\mu$ g/well (ELISA)	1/1000 dilution of HRP-conjugated rabbit anti-rabbit Ig	Chris Plumptre, Glaxo-Wellcome, Stevenage, U.K.
G30 (anti-C-terminus A $\beta$ )	0.5 $\mu$ g/well (ELISA)	1/1000 dilution of HRP-conjugated rabbit anti-rabbit Ig	Chris Plumptre, Glaxo-Wellcome, Stevenage, U.K.
G33 (anti-C-terminus A $\beta$ 42)	1 $\mu$ g/well (ELISA)	1/1000 dilution of HRP-conjugated rabbit anti-rabbit Ig	Chris Plumptre, Glaxo-Wellcome, Stevenage, U.K.

### 2.1.6 Primers

All oligonucleotide primers were constructed by Genosys, Pampisford, Cambs., U.K.

**Table VI: Sequences of primers**

Name	Sequence
APP forward	GTGCCAGCCAACACAGAAAACG
APP reverse	GGGTCTAGTTCTGCATCTGC
PS1 forward	GGTGTGGTGGGAATGATTTCC
PS1 reverse	CCATTCCTCACTGAACCCGCC

### 2.1.7 Animals

Lewis rats were obtained from breeding colonies within the Department of Biological Services, UCL or from Harlan Laboratories, Oxon, U.K.. Dead marmosets were obtained from the Department of Biological Services, King's College London. Aborted human fetuses were obtained from The Maudsley Hospital, King's College London.

### 2.1.8 Suppliers

Analytical grade general laboratory chemicals were obtained from Merck Ltd., (Poole, Dorset, U.K.), Boehringer Mannheim (Lewes, East Sussex, U.K.) or Sigma Chemical Company Ltd. (Poole, Dorset, U.K.). General disposable plasticware was supplied by Greiner (Stonehouse, Gloucester, U.K.) or Sterilin (Stone, Staffordshire, U.K.). Unless otherwise stated, additional laboratory materials were obtained from the following suppliers:

**Insight Biotechnology Ltd.**

4-chloro, 5-bromo, 3-indolyl- $\beta$ -galactosidase (X-Gal)

**Amersham International plc. (Little Chalfont, Bucks.)**

$\alpha$ -<sup>32</sup>P-dCTP (3000Ci/mM),  $\alpha$ -<sup>35</sup>S-dATP (400 Ci/mM), Rainbow™ coloured protein molecular weight markers, Hybond™-C and Hybond™-N membranes. Sequenase™ version 2.0 sequencing kit.

**Qiagen, Chatsworth, USA**

Qiagen “midi-prep” plasmid DNA extraction kits

**Bio-Rad (Hemel Hempstead, Herts.).**

Ammonium persulphate, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylene-diamine (TEMED)

**Dako Ltd. (High Wycombe, Bucks.).**

All fluorescein, rhodamine or peroxidase conjugated secondary antibodies.

**Difco Laboratories (Basingstoke, Hants.).**

Bacto-agar, Bacto-tryptone, Yeast extract.

**Gibco-BRL Life Technologies Ltd. (Renfrewshire, Scotland, U.K.)**

1 kb DNA ladder, all tissue culture and primary neuronal culture solutions, media and supplements.

**Nunc (Roskilde, Denmark)**

All tissue culture plasticware

**Promega Corporation (Madison, Wisconsin, USA).**

All restriction and modifying enzymes and buffers.

**Whatman International Ltd. (Maidstone, Kent, U.K.)**

3MM chromatography paper, Polydisc™ HD disposable 5µm filters

**Gelman Life Sciences (Ann Arbor, Michigan, U.S.A.)**

Disposable 0.45 and 0.2µm filters

**Pharmacia Biotechnology Ltd. (St. Albans, U.K.)**

Hexanucleotides [pd(N)<sub>6</sub>] for random prime labelling, dNTPs

**Sigma Chemical Company Ltd. (Poole, Dorset, U.K.)**

Kodak X-OMAT imaging photographic film, Kodak Professional 64T colour film.

**Cayla (Toulouse, France)**

Zeocin™ (phleomycin derivative)

**Millipore Ltd., Watford, U.K.**

Millicell™-CM culture plate inserts

**Southern Biotechnology Associates (Birmingham, Alabama, U.S.A.)**

Fluoromount G™, anti-neuronal specific tubulin antibody (TUJ1)

## **2.2 Molecular Biology**

### **2.2.1 Propagation of bacteria**

Bacteria were grown either in liquid LB media or on plates prepared from LB media containing 2% Bacto®-agar. Liquid cultures were grown overnight at 37°C in an orbital shaker at 200rpm. Bacterial plates were incubated overnight at 37°C in a standard incubator. Both media contained antibiotic selection as appropriate. Ampicillin was used at a final concentration of 100µg/ml. Tetracycline was used at a final concentration

of 12.5µg/ml in plates and 10µg/ml in liquid culture. Zeocin was used at a final concentration of 20µg/ml.

### **2.2.2. Transformation of bacteria**

Competent XL1-B or SCS110 cells were prepared using a standard calcium chloride technique (Sambrook *et al.*, 1989). A single bacterial colony was grown overnight in 10mls of LB containing no antibiotic. 100µl of this starter culture was used to inoculate 100mls of LB containing no antibiotic and the culture was grown to an OD<sub>580</sub> of approximately 0.5. The bacteria were then pelleted by centrifugation at 3000rpm for 10 mins and any excess LB was removed. The bacteria were resuspended in 10mls of ice-cold 100mM CaCl<sub>2</sub>, pelleted as before and resuspended in 4mls of ice cold CaCl<sub>2</sub>. The competent cells were then incubated on ice until required and used not more than 72 hours after preparation.

Competent cells were transformed by addition of DNA and subsequent incubation on ice for 30minutes. The cells were then heat shocked for 90 seconds at 42°C and returned to ice for a further 2 minutes. 800µl of LB was added and the cells incubated in an orbital shaker for 1 hour at 37°C/200rpm. The cells were then pelleted, resuspended in approximately 100µl of LB and plated onto LB agar plates containing the appropriate antibiotic selection. If detection of β-galactosidase activity was required, plates were previously prepared by spreading on 50µl of a 20mg/ml stock of X-Gal in dimethyl formamide (DMF).

### **2.2.3 Small scale plasmid DNA extraction from transformed bacteria**

The “mini-prep” extraction method used was based on a standard alkaline lysis protocol (Birnboim and Doly, 1979). Individual bacterial colonies were used to inoculate 3mls of LB containing the appropriate antibiotic selection. Cultures were incubated overnight at 37°C/200rpm. The cells from 1.5ml of culture were pelleted by centrifugation in a bench top microcentrifuge at 13000 rpm for 2 minutes and resuspended in 100µl of resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA pH8, 100µg/ml RNase-A.

Bacteria were then lysed by addition of 200µl of lysis buffer (200mM NaOH, 1% (v/v) Triton X-100) and neutralised by the addition of 150µl of neutralisation buffer (3M sodium acetate pH5.5). The cell lysate was then centrifuged for 3 minutes at 13000rpm and the pelleted precipitate was removed and discarded. 500µl of isopropanol was then added to the supernatant which was then vortexed and centrifuged for 5 minutes at 13000 rpm to pellet the DNA. The supernatant was removed and the DNA was washed with 500µl of 70% ethanol, dried under vacuum and then resuspended in 100µl of double-distilled water (ddH<sub>2</sub>O) containing 20µg/ml RNase A. Plasmid DNA was stored at -20°C.

#### **2.2.4 Large scale plasmid DNA extraction from transformed bacteria**

A single colony from a bacterial plate or approximately 50µl of liquid bacterial culture was used to inoculate 400mls of LB containing the appropriate antibiotic selection. This was then incubated overnight at 37°C at 200rpm. 100mls of this overnight culture were then spun down at 3000rpm for 10 minutes. Plasmid DNA was then extracted using the Qiagen midi-prep kit following the manufacturers instructions. A typical yield of DNA using this method was 100µg, which was resuspended in 100µl of ddH<sub>2</sub>O.

#### **2.2.5 Cosmid DNA extraction from transformed bacteria**

A single colony from a bacterial plate or approximately 50µl of liquid bacterial culture was used to inoculate 400mls of LB containing the appropriate antibiotic selection. This was then incubated overnight at 37°C at not more than 200rpm. 200mls of this overnight culture were then spun down at 3000rpm for 10 minutes. Cosmid DNA was then extracted using the Qiagen plasmid extraction midi-prep kit using the adaptations for cosmids suggested by the manufacturers. A typical yield of DNA using this method was 50µg, which was resuspended in 50µl of ddH<sub>2</sub>O.

#### **2.2.6 Restriction enzyme digestion**

Restriction digests were performed on plasmids or cosmids either for DNA analysis or isolation of DNA fragments. Analytical digests were usually performed in 20µl total

volume containing either 2µl of mini-prep DNA or 0.5-1µl of midi-prep DNA (approximately 1 µg DNA). Not more than 0.1 volumes of restriction enzyme(s) were added and the buffer recommended by the manufacturer was used at 1x concentration. Digests were incubated for at least 1 hour at the appropriate temperature. The digested DNA was electrophoresed on a 1% agarose gel (see section 2.2.10) and bands visualised on a UV transilluminator.

Restriction digests for isolation of DNA fragments were carried out in a total volume of 100µl containing approximately 5µg of midi-prep DNA and not more than 0.1 volumes of restriction enzyme(s). The buffer recommended by the manufacturer was used at 1x concentration. Digests were incubated for 1-16 hours at the appropriate temperature.

#### **2.2.7 Repairing restriction enzyme cleaved overhangs**

When there were no compatible restriction sites for cloning, restriction enzyme cleaved overhangs were filled in (5' overhangs) or "chewed back" (3' overhangs) using T4 DNA polymerase. After restriction digest, 1µl of a 25mM stock of dNTPs (dATP, dCTP, dTTP, dGTP) and 15 units of T4 DNA polymerase were added directly to the reaction. This reaction was then incubated for 45-60 minutes at 37°C. If the DNA was to be subjected to further manipulations, the reaction was heat inactivated at 80°C for 20 minutes and then cooled on ice for 5 minutes prior to the addition of further enzymes.

#### **2.2.8 Phosphatase treatment of plasmid DNA**

When religation of vector ends was possible during insert/vector ligation, the vector DNA was treated with calf intestinal alkaline phosphatase (CIAP). Restriction enzyme/blunt ending reactions were made up to 400µl with a final concentration of 1x alkaline phosphatase buffer, 10 units of CIAP and ddH<sub>2</sub>O. The reaction was incubated at 37°C for not more than 30 minutes and the CIAP was then inactivated by incubation at 80°C for 20 minutes.

### **2.2.9 Phenol/chloroform extraction and precipitation of DNA**

To purify DNA after a restriction enzyme, blunt ending or phosphatasing reaction, the reaction mix was made up to 400µl with ddH<sub>2</sub>O and an equal volume of tris-equilibrated phenol was added. The mixture was vortexed, centrifuged at 13000 rpm for 2 minutes and the aqueous phase removed and re-extracted with one volume of chloroform/IAA. The aqueous phase was again removed and the DNA precipitated by addition of 0.1 volumes of 3M sodium acetate (pH5.5) and 2 volumes of -20°C 100% ethanol.

### **2.2.10 Agarose gel electrophoresis**

1% (w/v) agarose in 1x TAE gels were cast. Ethidium bromide was added to a final concentration of 0.5µg/ml. Approximately 0.1 volume of 10x loading buffer (1x TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. A DNA marker appropriate to the size of the fragments being detected was used, usually a 1 kilobase (kb) ladder. DNA was electrophoresed at approximately 120mA until the fragments were well separated. Bands were visualised on a UV transilluminator and photographed onto polaroid film.

When a DNA fragment was to be isolated from the gel, low melting point agarose was used and the gels were run no higher than 60mA. Gels were run until the required band was well separated from the rest of the DNA. DNA was visualised on a UV transilluminator and the required bands were quickly and carefully excised using a scalpel. DNA fragments in agarose slices were stored at -20°C until required.

### **2.2.11 Ligations of DNA**

Ligations were carried out in a total volume of 30µl. Gel fragments were melted at 80°C for 5 minutes and then added directly to the ligation without further purification. Reactions usually contained 5µl of each gel fragment, 1x ligase buffer and 1-3 units of T4 DNA ligase in ddH<sub>2</sub>O. Reactions were allowed to proceed for approximately 1.5 hours at room temperature. Following ligation, the reaction was heated to 80°C for 5

minutes and an equal volume of ddH<sub>2</sub>O was added. The reaction was then transformed into a volume of competent bacteria (section 2.2.2) appropriate to the type of cloning.

#### **2.2.12 DNA sequencing**

1µg of phenol/chloroform extracted plasmid DNA was sequenced using the Sequenase™ kit v2.0 according to the manufacturers instructions. The Sequenase kit utilises the dideoxy chain termination method of sequencing (Sanger *et al.*, 1977).

#### **2.2.13 Polyacrylamide gel electrophoresis**

Sequencing reactions were run on an 8% 19:1 acrylamide: bisacrylamide, 50% urea gel in 1x TBE. The gel was pre-run at 1600 volts for at least 20 minutes prior to loading the sequencing reactions. 5µl of each reaction was loaded and the gel was run at 1800 volts for a period of time appropriate to the distance of the required sequence from the primer. The gel was then immersed in fixing solution (10% (v/v) methanol, 10% (v/v) acetic acid) for 20 minutes and placed between 3MM Whatman paper and saran wrap to dry under vacuum at 80°C in a Biorad gel drier. The gel was then exposed to X-ray film overnight.

#### **2.2.14. Southern blot analysis of viral genomes**

Southern blots (Southern, 1975) were performed on viral DNA to confirm genome structures of recombinant HSV1 vectors. 10µl of a viral DNA preparation was digested overnight with the appropriate enzymes and buffers in a total volume of 50µl. 0.1µg of plasmid control DNA was digested in a total volume of 20µl. The digest reactions were directly loaded and electrophoresed on a 1% agarose gel as described in section 2.2.10.

#### **2.2.15. Transfer of DNA to nitrocellulose**

The DNA was visualised on a UV transilluminator and photographed against a fluorescent ruler. The gel was left on the transilluminator for further 2 minutes to nick the DNA. The gel was then placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45minutes. The gel was then transferred to neutralising solution (2M NaCl, 1M Tris pH5.5) for a further 45minutes. The gel was then placed upside down on a plastic



support which was covered in a triple layer of 3MM Whatman paper which was used as a wick placed in a reservoir of 20x SSC. A piece of Hybond N nylon membrane cut to the same size as the gel was pre-soaked in the neutralising solution and then carefully placed on the gel ensuring there were no air bubbles present. 10 pieces of 3MM Whatman paper, pre-soaked in 20x SSC, were placed on the nylon membrane and a stack of dry paper towels and a suitable weight were placed on top. Parafilm was used to isolate the wick from the paper towels. The DNA was then transferred by capillary action to the nylon membrane overnight.

The membrane was removed, washed in 6x SSC and then air dried for 30 minutes. The DNA was then cross-linked to the membrane using a UV Stratalinker 2400.

#### **2.2.16 Radiolabelling of DNA**

Fragments of DNA were radiolabelled with  $\alpha$ -[ $^{32}\text{P}$ ]-dCTP for use as probes in Southern blot analysis. The method used was based on the random prime labelling reaction previously described (Feinberg and Vogelstein, 1983).

Approximately 1 $\mu\text{g}$  of the DNA to be used as the probe was digested and run on a 1% low melting point agarose gel as described in sections 2.2.6 and 2.2.10 respectively. The required DNA fragment was then excised from the gel and 3 volumes of ddH<sub>2</sub>O were added. The gel slice was then heated to 100°C for 5 minutes and then snap cooled by placing on ice for 2 minutes. 7 $\mu\text{l}$  of the melted gel slice was then placed in a screw cap Eppendorf containing 10 $\mu\text{l}$  of oligolabelling buffer, 5 units of DNA polymerase large fragment (Klenow) and 50 $\mu\text{Ci}$  of  $\alpha$ -[ $^{32}\text{P}$ ]-dCTP in a total volume of 50 $\mu\text{l}$ . The labelling reaction was then incubated at 37°C for 1-2 hours. The reaction was then filtered through a Sephadex column (G25 or G50) to remove any unincorporated label. The radiolabelled DNA probe was then heated for 5 minutes at 100°C and snap cooled on ice for 2 minutes. The denatured probe was then added to the hybridisation solution.

**Oligolabelling Buffer (OLB):**

Solution O: 1.25M Tris HCl pH 8.0  
0.125M MgCl<sub>2</sub>

Solution A: 1ml Solution O  
18μl β-mercaptoethanol  
5μl 0.1M dATP  
5μl 0.1M dGTP  
5μl 0.1M dTTP

Solution B: 2M HEPES pH6.6

Solution C: 90 units/ml random hexamers [pd(N)<sub>6</sub>]  
Dissolved in TE pH8.0

Oligolabelling buffer was made by mixing A:B:C in a ratio of 100:250:150.

**2.2.17 Hybridisation**

The nylon membranes were pre-hybridised for at least 2 hours at 65°C with 30mls of pre-hybridisation solution (6x SSC, 5x Denhardt's reagent, 0.5% w/v SDS in ddH<sub>2</sub>O containing 100μg/ml of denatured herring sperm DNA (100 x Denhardt's reagent: 2% w/v bovine serum albumin, 2% Ficoll® (type 400), 2% w/v polyvinylpyrrolidone in ddH<sub>2</sub>O)). The volume of the pre-hybridisation solution was reduced to approximately 5mls prior to adding the denatured probe. The probe was hybridised to the membrane overnight at 65°C. The membrane was then washed twice for 10 minutes in 2x SSC/0.1% w/v SDS. The filters were then wrapped in cling film and exposed to X-ray film at -70°C.

## **2.3 Cell culture**

All cell lines were stored long term in liquid nitrogen and during culture were maintained at 37°C in either a 5% CO<sub>2</sub> incubator in a humidified atmosphere (for flasks) or on a rotary roller apparatus (for roller bottles). All manipulations of cells were carried out under sterile conditions using standard aseptic techniques.

### **2.3.1 Freezing and recovery of cell stocks.**

Cell stocks for long term storage were prepared by suspending the cells from one 175cm<sup>2</sup> flask in 1.8 ml of 8% (v/v) dimethylsulphoxide (DMSO), 30% FCS, 68% appropriate medium (see section 2.1.4). The freezing vials were slowly cooled to -70°C, then immersed in liquid nitrogen. Cells were recovered by rapidly thawing the contents of one vial and transferring the cells to a 25cm<sup>2</sup> flask of pre-warmed medium containing the appropriate selection if required. The medium was changed or the cells passaged the following day.

### **2.3.2 Routine cell passage**

Cells were grown in 175cm<sup>2</sup> flasks, 500cm<sup>2</sup> plates or 800cm<sup>2</sup> roller bottles in the appropriate growth media (see section 2.1.4). When they were 80-90% confluent the cells were passaged as follows. For BHK, BHK-based cell lines, HEK293, HEK293-based cell lines and vero cells, the monolayers were rinsed with Hank's Balanced Salt Solution (HBSS), detached with trypsin/versene (1:10) and disaggregated. Fresh flasks were seeded at a ratio of 1:10 (BHK), 1:6 (vero) or 1:3 (HEK293). Roller bottles were gassed with CO<sub>2</sub> to give a final concentration of 5%.

### **2.3.3 Transfection of plasmids for transient expression**

BHK or BHK-based cells were transiently transfected with 5-10µg supercoiled plasmid DNA per well of a 6-well plate. The protocol was based on the standard calcium phosphate transfection method (Stow and Wilkie, 1976).

HEBES Transfection Buffer:      140mM NaCl  
   5mM KCl  
   0.7mM Na<sub>2</sub>HPO<sub>4</sub>  
   5.5mM D-glucose  
   20mM Hepes  
   pH 7.05 with NaOH  
   Filter sterilised with a 0.2µm filter. Stored at 4°C

BHK or BHK-based cells were grown until they were at 70% confluency.

Two tubes were set up:

Tube A: 31µl 2M CaCl <sub>2</sub>	Tube B: 400µl HEBES transfection buffer
10µg plasmid DNA	
20µg herring sperm DNA (phenol/chloroform extracted)	

The contents of tube A were thoroughly mixed and then added slowly to tube B whilst it was being continually vortexed. The mixture was then left for 20-40 minutes at room temperature to allow the DNA to precipitate. Media was removed from the cells and the precipitated DNA mixture was slowly added. The cells were then incubated for 20-40 minutes at 37°C. 1ml of growth media without selection was then added to the cells and the plate incubated for 4 hours at 37°C. The media was then removed and the cells washed twice with 2mls of growth media. 1ml of 25% (v/v) DMSO in HEBES transfection buffer was then added and left on the cells for no longer than 2.5 minutes. The DMSO solution was removed and the cells immediately and quickly washed twice with 2mls of growth media. A final 2mls of growth media without selection was added to the cells which were then incubated at 37°C/5% CO<sub>2</sub> for 24 or 48 hours.

#### **2.3.4 Detection of β-galactosidase expression by X-Gal staining**

Media was removed and the cells were washed twice with 2mls of 1x PBS. The cells were then fixed in 1ml of 1x PBS containing 0.05% glutaraldehyde for 10 minutes at room temperature. The cells were then washed twice with 2mls of 1x PBS and

incubated at 37°C for 1-16 hours in 3mls/well of X-Gal stain (5mM  $K_3Fe(CN)_6$ , 5mM  $K_4Fe(CN)_6 \cdot 6H_2O$ , 1mM  $MgCl_2$ , 150 $\mu$ g/ml X-Gal in DMSO in 1x PBS). The X-Gal stain was then removed and replaced with 70% (v/v) glycerol for storage.

### **2.3.5 Detection of GFP expression**

Cells expressing GFP required no pre-treatment and were visualised directly under an inverted fluorescent microscope at a wavelength of 500nm.

### **2.3.6 Determination of antibiotic resistance threshold (“killing curve”)**

Cells were plated in 6-well plates and grown to approximately 70% confluency (except if zeocin was the antibiotic to be used, when cells were grown to 25% confluency). The appropriate antibiotic was applied at a range of concentrations around that recommended by the manufacturer. The selective media was changed every 2-3 days and the minimum concentration of antibiotic required to cause complete cell death in 5 days was determined.

### **2.3.7 Construction of stable cell lines**

Stable cell lines were constructed by an adaptation of the transfection protocol detailed in section 2.3.3. 10 $\mu$ g of plasmid DNA was linearised, phenol/chloroform extracted and resuspended in 10 $\mu$ l ddH<sub>2</sub>O, all of which was used in one transfection. The protocol was then carried out exactly as described in section 2.3.3, with the exception that the cells to be transfected were grown in 100mm wells and the volumes of other reaction products were scaled up accordingly (89 $\mu$ l 2M  $CaCl_2$ , 10 $\mu$ l HS DNA, 1150 $\mu$ l HEBES transfection buffer). After the DNA precipitate has bound to the cells, 3mls of growth media was added and after the DMSO shock, 10mls of growth media was added. The day after transfection, the cells were washed and the selective media was applied at the concentration determined by the killing curve (section 2.3.6). If transfected cells were to be selected by zeocin resistance, the 100mm wells were split 1:15 24 hours after transfection and the selective media applied to all wells 24 hours after the split. This extra step was necessary as zeocin is able to kill unresistant cells most efficiently when the monolayer is not more than 25% confluent.

The selective media was changed every 2-3 days until colonies were clearly visible but still well isolated. Colonies were picked under an inverted microscope and transferred to 96-well plates. When 80-90% confluent, the cells were split and grown through 24 and 6-well plates with changing of the selective media every 2 days. Cells were always moved before they became 100% confluent. Cell lines were then transferred to 25cm<sup>2</sup> flasks and 2 ampoules of each were frozen down by the method described in section 2.3.1. At this stage, 24-well plates of all cell lines were set up to enable screening of the cells for the ability to grow disabled HSV1.

### **2.3.8 Screening of cell lines**

Each cell line was split into 2 wells of a 24-well plate and grown to 80% confluency. The wells were infected at a multiplicity of infection of 0.01 with an appropriately disabled virus. The virus was harvested and titred 48 hours post infection (see section 2.4.2). A small number of the best cell lines were selected for further analysis by growth curves (see section 2.4.8). The optimal cell line was then selected and cloned out.

### **2.3.9 Obtaining a pure population of cells**

Once the optimal cell line had been identified, this was cloned out by limiting dilution in a 96-well plate and slowly amplified under continual selection. A large number of 175cm<sup>2</sup> flasks of the pure cell line were grown and frozen down. These frozen ampoules were stored in liquid nitrogen and a new one was defrosted every 4-6 weeks (see section 2.3.1)

## **2.4 Virus construction and propagation**

### **2.4.1 Homologous recombination into the HSV1 genome**

Transfections were carried out as described in section 2.3.3 except 10-30µg of purified viral DNA (see section 2.4.7) was added to tube A and tubes were mixed much more gently at each stage. The DNA precipitate was left on the cells for 7 hours prior to the DMSO shock. For transfections using viral DNA from HSV1 mutants with a disrupted VP16 gene, the media was supplemented with 3mM hexamethylene bisacetamide

(HMBA) in order to induce immediate early gene transcription (McFarlane *et al.*, 1992). Homologous recombination transfections were left for 3-5 days until complete cytopathic effect (CPE) was observed. The well was then harvested and freeze-thawed. The harvested cells were then titred out (see section 2.4.2) and the efficiency of the recombination determined by assaying for loss or gain of a reporter gene.

#### **2.4.2 Viral infectivity (plaque) assay**

Serial ten fold dilutions of the virus were prepared in DMEM without FCS and plated onto 80% confluent wells of BHKs or the appropriate complementing cell line. A minimum volume of 100µl/well was used in 24-well plates and 500µl/well in 6-well plates. The virus was allowed to adsorb for 40-60 minutes at 37°C/5%CO<sub>2</sub> and then the monolayers were overlaid with 2mls of a 1:2 (v/v) mix of 1.6% (w/v) carboxymethyl cellulose:growth media without selection (supplemented with 3mM HMBA if necessary). The cells were then incubated for a further 48 hours at 37°C/5%CO<sub>2</sub> and the number of plaques in each well were counted in order to determine the titre of the virus in plaque forming units (pfu)/ml.

#### **2.4.3 Purification of viral recombinants by plaque selection**

Harvested wells from homologous recombination transfections were freeze-thawed to disrupt the cells and titred out as described in section 2.4.2. If the transfection was designed to result in the loss or gain of *lacZ*, then the plaques were stained as described in section 2.3.4 except the cells were not fixed. If the transfection was designed to result in the loss or gain of GFP, the plaques were directly visualised under an inverted fluorescent microscope. Recombinants were identified as blue/green/white plaques as appropriate and were picked from the monolayer in a minimum volume (less than 2µl) using a P20 Gilson micropipette. Plaques were transferred to an eppendorf containing 100µl of DMEM without FCS and freeze-thawed to disrupt the cells. 10µl and 90µl of each plaque suspension was then used to infect 2 wells of a 6-well plate of the appropriate complementing cell line at 80% confluency. The virus was allowed to adsorb for 40-60 minutes at 37°C/5%CO<sub>2</sub> and then the monolayers were overlaid with 2mls of a 1:2 (v/v) mix of 1.6% (w/v) carboxymethyl cellulose:growth media without

selection (supplemented with 3mM HMBA if necessary). When a very disabled virus was being purified away from a less disabled backbone, 3% low melting point agarose in ddH<sub>2</sub>O was used in place of the carboxymethyl cellulose. The cells were then incubated for a further 48 hours at 37°C/5%CO<sub>2</sub>. The plaque purification process was repeated until a pure population was obtained. The whole well was then harvested and used as a starting stock for large scale propagation of the recombinant virus.

#### **2.4.4 Production of high titre stock of recombinant virus**

For less disabled viruses (those which have either one or no essential IE genes deleted), 10 x 850cm<sup>2</sup> roller bottles (Corning Glass Works, Corning, New York, USA) were seeded with the appropriate complementing cell line in 100mls of growth media without selection. The cells were grown to 90% confluency and infected at an MOI of 0.01 (approximately 1 x 10<sup>6</sup>pfu of virus per roller bottle) in a total volume of 100mls of new growth media without selection (supplemented with 3mM HMBA if necessary). The roller bottles were then incubated at 32°C/0.5rpm for 3-5 days until complete CPE was observed. The cells and supernatant were harvested by vigorous shaking of the roller bottle and immediately frozen at -80°C.

For more disabled viruses (those with both essential IE genes deleted), 10 x 500cm<sup>2</sup> plates were seeded with the appropriate complementing cell line in 50mls of growth media without selection. The cells were grown to 90% confluency and infected at an MOI of 0.05 (approximately 3 x 10<sup>6</sup>pfu of virus per plate) in a total volume of 50mls of new growth media without selection (supplemented with 3mM HMBA if necessary). The plates were then incubated at 37°C for 2-4 days until complete CPE was observed. The whole plate was then transferred directly to -80°C.

After defrosting, the cellular debris was removed by centrifugation at 3500rpm for 45 minutes at 4°C. The supernatant was immediately decanted and filtered through a 5µm and then a 0.45µm filter and the virus pelleted by centrifugation at 12000rpm for 2 hours at 4°C. The supernatant was then discarded and the viral pellet was gently resuspended in DMEM without any supplements. The resuspension volume depended on the degree of disablement of the virus but was generally 1-3mls/10 roller bottles (for the less disabled viruses) or 500µl/10 plates (for the more disabled viruses). The



resuspended viral pellet was then sonicated for 5 x 10 seconds in a water bath sonicator with chilling on ice between sonications. Aliquots of the virus were stored in liquid nitrogen and the titre of a freeze-thawed aliquot was determined using the standard viral infectivity assay (see section 2.4.2).

#### **2.4.5 Small scale viral DNA extraction**

Small-scale viral DNA extractions were performed in order to obtain viral DNA for Southern blot analysis. The method was based on that described by Feldman, 1996. One well of a 6-well plate of virally infected cells at complete CPE were harvested and pelleted by centrifugation at 100rpm for 10minutes. The cells were resuspended in 200µl of TES buffer (50mM Tris pH7.8, 1mM EDTA, 30% (v/v) sucrose). 200µl of Proteinase K buffer (2% (w/v) SDS, 100mM β-mercaptoethanol) was added and the reaction incubated on ice for 30mins. 10µl of Proteinase K (stock at 20mg/ml in 10mM CaCl<sub>2</sub>) was then added and the reaction was incubated at 55°C overnight. Extractions were performed twice using phenol/chloroform (1:1 v/v) and then once with chloroform/isoamyl alcohol (24:1 v/v). Viral DNA was precipitated by the addition of 75µl of 7.5M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol and pelleted by microcentrifugation at 13000rpm for 10minutes. The DNA pellet was then washed with 500µl of 70% EtOH and air dried. Viral DNA was then resuspended in 20-50µl of ddH<sub>2</sub>O, depending on the degree of disablement of the virus.

#### **2.4.6 Large scale viral DNA extraction**

Large-scale viral DNA extractions were carried out for use in homologous recombination transfections. Virally infected cells at complete CPE were harvested from 8 x 175cm<sup>2</sup> flasks. The harvested stock was then centrifuged for 2 hours at 12000rpm at 4°C in order to pellet the cells and virus. The pellet was then transferred into 15mls of Proteinase K buffer (0.01M Tris pH8.0, 5mM EDTA, 0.5% SDS) and Proteinase K (Boehringer Mannheim) was added to a final concentration of 50µg/ml. The reaction was then incubated at 37°C and 200rpm overnight. 15mls of ddH<sub>2</sub>O were then added to the lysate. Extractions were performed by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1(v/v)) followed by gentle mixing by

inversion for 10 minutes and subsequent centrifugation at 15000rpm for 10 minutes. The aqueous layer was extracted 3 times or until no white interface appeared after centrifugation. A final extraction was performed using an equal volume of chloroform/isoamyl alcohol (24:1 (v/v)). Two volumes of ethanol were gently layered onto the aqueous solution and the layers slowly mixed by inversion. The precipitated viral DNA was then pelleted by centrifugation at 3000rpm for 10 minutes. The viral DNA was then washed with 5mls of 70% ethanol and the pellet was then air dried overnight. The pellet was then resuspended in 0.5-3mls of ddH<sub>2</sub>O depending on the degree of disablement of the virus. The integrity and approximate concentration of the DNA was determined by running 5µl on a 1% agarose gel.

#### **2.4.7 Growth curves**

80% confluent 24-well plates of the appropriate complementing cell line were infected at an MOI of 0.1 or 0.01 in a final volume of 100µl DMEM without FCS. Virus was allowed to adsorb for 40-60 minutes at 37°C/5%CO<sub>2</sub> and then the monolayers were overlaid with 500µl of growth media without selection (supplemented with 3mM HMBA if necessary). Cells were harvested at 0, 4, 8, 16, 24 and 36 hours post infection. Harvested samples were freeze-thawed to disrupt the cells and yield of virus was measured by plaque assay (see section 2.4.2).

### **2.5 Protein extraction and analysis**

#### **2.5.1 Standard protein extraction from cultured cells**

1 x 10<sup>6</sup> cells were washed twice in 1ml of 1x PBS and harvested in 100µl of standard protein sample buffer (5% β-mercaptoethanol, 50mM Tris-HCl pH 8.0, 6% (v/v) glycerol, 2% (w/v) SDS, and 0.005% (w/v) bromophenol blue). The samples were immediately placed on ice and then heated to 95°C for 5 minutes. The samples were either loaded immediately onto a SDS-polyacrylamide gel (see section 2.5.3) or stored at -20°C.

### **2.5.2 Extraction of multiple TM spanning or “fragile” proteins from cultured cells**

$1 \times 10^6$  cells were washed twice in 1ml of 1x PBS and harvested in 1ml 1x PBS. Cells were pelleted at 2000rpm for 3 minutes in a microcentrifuge and the pellet resuspended in 100 $\mu$ l of 2x extraction buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 1mM PMSF, 10 $\mu$ g/ml leupeptin and 10 $\mu$ g/ml pepstatin). The resuspended cells were sheared by passing through a 21 gauge needle 10 times. The samples were either stored at –20°C at this stage or further processed as follows. 100 $\mu$ l of 2x TM protein sample buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 350mM  $\beta$ -mercaptoethanol) was added and the sample incubated at 37°C for 15 minutes prior to loading onto a SDS-polyacrylamide gel containing 2M urea (see section 2.5.3). This protocol was developed for the purposes of this thesis and was based on suggestions from Paul Mathews, Nathan Kline Institute, New York University. The protocol was specifically designed for the extraction of presenilin 1 which was found not to resolve clearly using the method described in section 2.5.1 or standard adaptations thereof. The protocol has since been found to offer better resolution of other “fragile” proteins on SDS-polyacrylamide gels (for example HSV1 ICP4).

### **2.5.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein samples were heated to 95°C for 5 minutes or 37°C for 15 minutes depending on the extraction procedure (see sections 2.5.1 and 2.5.2). Approximately 10 $\mu$ l of sample or 3 $\mu$ l of coloured molecular weight protein standards (rainbow markers) were loaded/lane. SDS-polyacrylamide gels were prepared and run in a vertical gel electrophoresis system according to the method of (Laemmli, 1970). The composition of the stacking and resolving gels was as shown in table VII.

**Table VII: Composition of stacking and resolving gels used in SDS-PAGE**

	4x resolving gel buffer pH 8.8	4x stacking gel buffer pH 6.8
Tris base	36.3 g	12.1 g
SDS	0.8 g	0.8 g
ddH <sub>2</sub> O to	200 ml	200 ml

Both buffers were adjusted to the correct pH with conc. HCl.

	Resolving gel			Stacking gel
	15%	10%	7.5%	5%
Acrylamide-bis*(30:1.5)	15.75	10.5 ml	7.9 ml	1.6 ml
4 x buffer	7.9 ml	7.9 ml	7.9 ml	2.6 ml
10% APS	150 µl	150 µl	150 µl	100 µl
TEMED**	9 µl	9 µl	9 µl	5 µl
ddH <sub>2</sub> O to	32.5 ml	32.5 ml	32.5 ml	10.55 ml

\*Bis = N,N'-Methylene-bis-acrylamide

\*\* TEMED = NNNN-tetraethylethanediamine

When the extraction method described in section 2.5.2 was used, both the resolving and stacking gels contained 2M urea.

Gels were run at constant current (30-40 mA/gel) with variable voltage in 1x running buffer (25mM Tris, 250mM glycine and 0.1% (w/v) SDS, pH 8.3). Gels were run until the protein of interest would be in approximately the middle of the gel (as determined by the migration of the coloured molecular weight markers).

#### **2.5.4 Equalisation of protein loading**

Duplicate protein samples were separated by SDS-PAGE (section 2.5.3) and the gel placed in Coomassie stain solution (2% (w/v) Coomassie brilliant blue R250, 50% (w/v) methanol, 50% (v/v) glacial acetic acid) for 1 hour at room temperature with continual shaking. Any unbound stain was then removed by repeated replacements of destain solution (10% (w/v) glacial acetic acid, 30% (v/v) methanol).

#### **2.5.5 Transfer of proteins to nitrocellulose membranes (western blotting)**

Proteins separated on SDS-PAGE gels were transferred to Hybond C membranes using a wet-transfer method based on that of (Towbin *et al.*, 1979). Briefly, the SDS-PAGE gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 380mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol), sandwiched between sheets of pre-soaked Whatman 3MM paper, and a Trans-Blot™ Cell (BioRad) assembled according to manufacturer's instructions. Transfer was carried out overnight at 200mA and 4°C. Following transfer, protein bands were viewed by staining the membrane with 0.5% (w/v) Ponceau S (in 1% (v/v) acetic acid) then washing the membrane with several changes of 1x PBS.

#### **2.5.6 Immunodetection of proteins on western blots**

Following transfer, the membranes were blocked by incubation in 5% (w/v) skimmed milk powder in 1x PBS for 1 hour at room temperature with constant shaking. The membrane was then incubated with primary antibody diluted in 3% (w/v) skimmed milk powder in 1x PBS for 1-2 hours at room temperature with constant shaking. The antibodies used in this thesis, their sources and the dilutions they were used at are listed in table V which can be found in section 2.1.5. Unbound antibody was removed by washing the membrane for 3 x 10 minutes in 1x PBS with 0.1% Tween-20 (PBST) at room temperature with constant shaking. The membrane was then incubated in an appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody diluted in 3% (w/v) skimmed milk powder in 1x PBS for 1 hour at room temperature with constant shaking. Secondary antibodies were diluted as stated in table V, section 2.1.5. Unbound antibody was removed by washing the membrane for 3 x 10 minutes in

1x PBST at room temperature with constant shaking. The bound HRP was then detected using an enhanced chemiluminescence system (ECL™) according to the manufacturer's instructions. The membrane was then exposed to X-ray film to detect the resultant light emissions. Exposure times were between 1 second and 15 minutes, depending on the strength of the signal.

### **2.5.7 Immunofluorescence**

Cell monolayers or primary cultures were washed twice with 1x PBS. Cells were either fixed in ice-cold methanol for 15 minutes or incubated for 10 minutes at room temperature with 4% paraformaldehyde (in water). The fixative was replaced with 0.1% (v/v) Triton X-100 in 1x PBS and incubation was continued for a further 5 minutes. Cells were treated with 5% (v/v) normal goat serum (NGS) in 1x PBS for 10 minutes and then washed 3 times with 1x PBS. The cells were then incubated with primary antibody diluted in 1x PBS for 1 hour at room temperature, then washed 3 times with 1x PBS. An appropriate fluorescein isothiocyanate (FITC)-conjugated or rhodamine-conjugated anti-IgG was added and incubation continued for a further hour at room temperature in the dark. Cells in tissue culture plates were viewed directly under an inverted fluorescent microscope. Coverslips were inverted and mounted in fluoromount G™ and cells viewed using a fluorescent microscope.

### **2.5.8 Enzyme linked immunosorbant assay (ELISA)**

ELISAs were carried out to assess levels of secreted  $\beta$ -amyloid protein. The  $\beta$ -amyloid ELISA used was developed by the Neurosciences Unit, Glaxo-Wellcome. A Nunc immunosorb plate was coated with the capture antibody in 100 $\mu$ l/well of carbonate/bicarbonate buffer (1.6g Na<sub>2</sub>CO<sub>3</sub>, 2.9g NaHCO<sub>3</sub> made up to 500mls with ddH<sub>2</sub>O, pH 9.5). The plate was incubated overnight at 4°C. Unbound antibody was removed by washing the plate three times in 1x PBS and the plate was then blocked in 200 $\mu$ l 3% (w/v) BSA in 1x PBS and 0.05% (w/v) sodium azide overnight at 4°C. Samples and standards were diluted in 1x EC buffer (0.02M phosphate buffer, pH7.0, 0.4M NaCl, 2mM EDTA, 0.4% (w/v) skimmed milk powder, 0.2% (w/v) BSA, 0.05% (v/v) CHAPS, 0.05% (w/v) sodium azide) and 100 $\mu$ l of sample or standard was

added/well of the ELISA plate. The plate was again incubated overnight at 4°C and unbound sample removed by washing three times in PBST. Biotinylated secondary antibody diluted in buffer C (0.02M phosphate buffer, pH 7.0, 0.4M NaCl, 2mM EDTA) was added at 100µl/well and the plate incubated again overnight at 4°C. Unbound antibody was removed by washing three times in 1x PBST. 100µl/well of streptavidin-HRP in 1x PBST was added to each well and the plate incubated for 1 hour at room temperature. The plate was washed three times with 1x PBST and the signal detected using 100µl TNB/well. Once the colour had developed, the reaction was stopped by addition of 100µl/well H<sub>2</sub>SO<sub>4</sub> and the plate was read at 450nm. The dilutions and sources of the capture and secondary antibodies used in the ELISAs are listed in table V, section 2.1.5.

### **2.5.9 Preparation of samples for caspase assay**

Neurons were washed twice in 1x PBS and harvested in caspase extraction buffer (10 mM HEPES pH 7.4, 50mM KCl, 2mM MgCl<sub>2</sub>, 5mM EGTA, 0.1% (v/v) CHAPS, 1mM DTT, 1mM PMSF, 10µg/ml of leupeptin, pepstatin A and aprotinin). Approximately 200-250µg of protein was extracted in 60µl of this buffer by vortexing or homogenising the harvested neurons.

## **2.6 *In vivo* gene delivery and primary neuronal cultures**

### **2.6.1 Dissociated primary neuronal cultures**

#### **2.6.1.1 Preparation of neuronal cultures of adult rat dorsal root ganglia (DRG)**

An adult rat was injected with a lethal dose of Expiral™ (Sanofi Animal Health, Watford, U.K.) and decapitated. The spinal column was removed and as many DRGs as possible dissected out and transferred to ice-cold DMEM. Excess non-neuronal tissue was removed and the DRGs were digested with 0.25% collagenase A at 37°C for 30-45 minutes. Remaining clumps of tissue were removed by filtering through a 70µm cell strainer and the neurons were resuspended in DMEM supplemented with 10% (v/v)

horse serum. Neurons were plated on coverslips in 24-well plates which had been pre-treated with 100ng/ml poly-DL-ornithine in 0.15M sodium borate buffer pH8.4, thoroughly washed and coated in 5µg/ml laminin in 1x PBS. Half the media was changed every second day and the adult rat DRG neurons were cultured for 1 week prior to infection with recombinant virus. This protocol was carried out in collaboration with James Palmer, UCL.

#### **2.6.1.2 Preparation of cortical neurons from E18 rat**

Primary cultures of E18 rat cortical neurons were prepared by Liz Ensor at the Institute of Child Health, UCL. Half the growth media (MEM with Earle's Salts without glutamine supplemented with 10% (v/v) horse serum and 4mg/ml D-glucose) was changed every second day and the neurons were cultured for 24 hours to 1 week prior to infection with recombinant virus.

#### **2.6.1.3 Preparation of primary neurons from 12-20 week aborted human fetuses**

Primary cultures of human fetal neurons were prepared by Ritchie Williamson at the Institute of Psychiatry, King's College London. The growth media (Neurobasal™ with 1% (v/v) B27 serum free supplement) was changed every second day and the neurons were cultured for 2.5 weeks prior to infection with recombinant virus.

#### **2.6.1.4 Infection of dissociated primary cultures**

The growth media was removed from the neurons and replaced with a minimum volume of DMEM without any supplements. Recombinant virus was added at an MOI of between 1 and 5. The virus was allowed to adsorb for 40-60 minutes at 37°C/5%CO<sub>2</sub> and then the neurons were overlaid with 500µl of growth media and incubated at 37°C/5%CO<sub>2</sub>. Growth media was changed every 2-3 days and the infected neurons were maintained for up to a month. Cells were stained for β-galactosidase expression, visualised for GFP expression or processed for immunofluorescence as described in sections 2.3.4, 2.3.5 and 2.3.7 respectively.



## **2.6.2 Organotypic neuronal cultures**

### **2.6.2.1 Preparation of organotypic hippocampal slice cultures**

The culturing methods used are a modification of those previously described (Stoppini *et al.*, 1991). Brains were removed following rapid decapitation and placed in ice-cold dissecting media (DMEM, 200U/ml penicillin and 200mg/ml streptomycin). After hemisecting the brain, the hippocampus was isolated by making a dorso-rostral cut through the fornix, a ventro-caudal cut through the entorhinal cortex and the freed hippocampus was rolled out over the neocortex. Hippocampi were placed dorsal side up on a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co, Ltd., Gomshall, Surrey, UK) and 300 µm slices were made in the coronal plane. The hippocampal slices were then immediately transferred to pre-warmed growth media (50% (v/v) MEM, 25mM HEPES, 25% (v/v) horse serum, 25% (v/v) HBSS, supplemented with 2mM L-glutamine and 6.4mg/ml D-glucose). The slices were carefully separated and transferred onto pre-warmed 30 mm Millicell™-CM culture plate inserts with 1ml of culture media placed below the membrane. 3 to 4 slices were placed on each insert. Cultures were maintained in a humidified incubator at 37°C with 5 % CO<sub>2</sub> and the culture media was changed every 2 days. Slices were cultured for 1 week prior to infection with recombinant virus.

### **2.6.2.2 Infection of organotypic hippocampal slice cultures**

Organotypic hippocampal slices were infected with  $1 \times 10^6$  pfu of recombinant HSV1. Slices were infected in 2mls of mRoth media (50% (v/v) MEM, 25mM HEPES 2% (v/v) TCM™ Supplement (ICN Biomedicals), 25% (v/v) HBSS, supplemented with 2mM L-glutamine and 6.4mg/ml D-glucose). The culture media was removed from below the slices and the virus suspension in mRoth was slowly added on top of the slices. The slices were then incubated at 37°C/5%CO<sub>2</sub> for 1 hour. The media was then removed and 1ml of growth media was placed below the insert membrane. Growth media was changed every 2-3 days and the infected neurons were maintained for up to a month.

### **2.6.2.3 X-Gal staining of organotypic hippocampal slices cultures**

$\beta$ -Galactosidase activity was determined by histochemical staining of the whole slice with X-Gal. The culture media was removed from the wells, slices were washed in 1x PBS, fixed in 0.5% (v/v) glutaraldehyde for 15 minutes, washed twice again in 1x PBS and transferred to hippocampal slice X-Gal staining solution (5mM  $K_3Fe(CN)_6$ , 5mM  $K_4Fe(CN)_6 \cdot 6H_2O$ , 1mM  $MgCl_2$ , 0.02% (w/v) sodium deoxycholate, 0.02% (v/v) NP40, and 750 mg/ml X-Gal (dissolved in DMSO) in 1x PBS). Slices were incubated in X-Gal staining solution overnight at room temperature.

### **2.6.2.4 Detection of GFP expression in organotypic hippocampal slice cultures**

The detection of GFP expression in the organotypic hippocampal slice cultures required no pre-treatment of the slices. The insert membranes containing the slices were placed in 60mm dishes containing a small amount of the culture media. The slices were then visualised on a fluorescent microscope and photographed.

## **2.6.3 *In vivo* gene delivery**

### **2.6.3.1 Animal surgery**

220g female Lewis rats were anaesthetised and positioned in a stereotaxic apparatus. Rats were injected in the striatum with  $2.5 \times 10^5$  pfu (5 $\mu$ l of a  $5 \times 10^7$  pfu/ml stock) of recombinant virus over 10 minutes. At various time points post injection, animals were perfused fixed in 4% ice-cold paraformaldehyde and the brains removed. Animal surgery was carried out by Zi-Qun Han, Department of Molecular Pathology, UCL

### **2.6.3.2 Sectioning and processing of brains**

Brains were post-fixed for 2 hours and 200 $\mu$ m sections were cut in the parasagittal plane on a Vibroslice™ (Campden instruments, Sileby, Leics.). GFP expression was visualised directly on a fluorescent microscope.  $\beta$ -galactosidase expression was detected by transferring the slices to tissue X-Gal stain (5mM  $K_3Fe(CN)_6$ , 5mM  $K_4Fe(CN)_6 \cdot 6H_2O$ , 1mM  $MgCl_2$ , 0.02% (w/v) sodium deoxycholate, 0.02% (v/v) NP40, and 40mg/ml X-Gal (dissolved in DMSO) in 1x PBS). Slices were incubated overnight

in a humidified atmosphere at 37°C without 5% CO<sub>2</sub>. The X-Gal solution was then removed and the slices placed in 70% (v/v) glycerol and stored at 4°C prior to photographing.

## **CHAPTER 3**

# **CONSTRUCTION OF INITIAL VIRUSES CONTAINING BICISTRONIC REPORTER GENE CASSETTES**

### 3.1 Introduction

The HSV1 latency associated transcripts (LATs) are the only viral gene products expressed in latently infected neurons. As discussed in chapter 1, the potential to harness this ability to continue to express genes throughout latency is one of the motivations behind using HSV1 as a gene delivery vector. In order to successfully exploit latency in this way, it is necessary to identify the regulatory elements which control the stable expression of the LATs during the normal course of a latent infection.

As discussed in the general introduction, expression of the LATs is controlled by two promoters, LAP1 and LAP2. LAP1 is located upstream of the region coding for the unstable 8.3kb LAT and contains basal promoter elements, including a TATA box, ATF/CREB and USF-1 sites (Soares *et al.*, 1996; Bloom *et al.*, 1997). LAP2 is a TATA-less promoter located immediately 5' of the stable 2kb LAT intron (Goins *et al.*, 1994). The positions of LAP1 and LAP2 are shown in figure 3.1.1. A number of attempts have been made to delimit the regions of LAP1 and/or LAP2 that are required to facilitate continued expression through latency. These studies were discussed in detail in the general introduction. Briefly, it has been found that LAP1 is not sufficient to drive long term expression and that downstream elements (within and/or surrounding LAP2) are required (Lachmann and Efsthathiou, 1997; Lokensgard *et al.*, 1997; Berthomme *et al.*, 2000).

Furthermore, it has been reported that LAP2, but not LAP1 is capable of independently expressing transgenes through latency from ectopic locations in the viral genome *in vivo*. (Goins *et al.*, 1994). In this study, the LAP2 promoter was used to drive expression of  $\beta$ -galactosidase in the glycoprotein C locus, a site far removed from the LAT region. Although the data was not shown, the authors reported LAP2 driven expression of the transgene for 300 days. However, this observation was somewhat hampered by the fact that the levels of transgene expression afforded by the LAP2 promoter were very low and the authors had to use RT-PCR to detect the *lacZ* mRNA (Fink *et al.*, 1996). Nevertheless, this report does demonstrate the potential of using LAT-derived promoters to drive long term expression at sites away from the LAT region.

Investigations of the structure of the LAP2 promoter have revealed a number of interesting properties. As a promoter without a TATA box, LAP2 is reliant on TFIID independent mechanisms to facilitate gene expression. Several potential *cis* acting elements have been identified which may be responsible for increasing the promoter activity of LAP2. These include an Sp1 site and putative AP2 and E2F sites (Goins *et al.*, 1994). LAP2 also contains CT rich and polyT elements which bind certain transacting factors known to regulate cellular housekeeping gene promoters. However, whether binding of such factors is of *in vivo* relevance in the HSV1 lifecycle has yet to be proven. An example of cellular factors which can directly bind to LAP2 are the group of small chromosomal chromatin-associated proteins known as HMG (high mobility group) proteins (French *et al.*, 1996). This observation may be relevant to the role of LAP2 in latency because HMG proteins are known to only be found in active chromatin, where they are thought to bind to nucleosomes and uncoil them, making the DNA available as a template for RNA synthesis. Furthermore, the LAT regions of HSV1 have an altered dinucleotide content compared to the rest of the genome, despite remaining similarly GC rich. It has been proposed that this altered dinucleotide content may reflect an altered local DNA structure in this region during latency (Coffin *et al.*, 1995). It could therefore be imagined that the unusual secondary structure of LAP2 and surrounding regions might be an important factor in enabling this area of the genome to escape the shut off of transcription that occurs at the onset of latency.

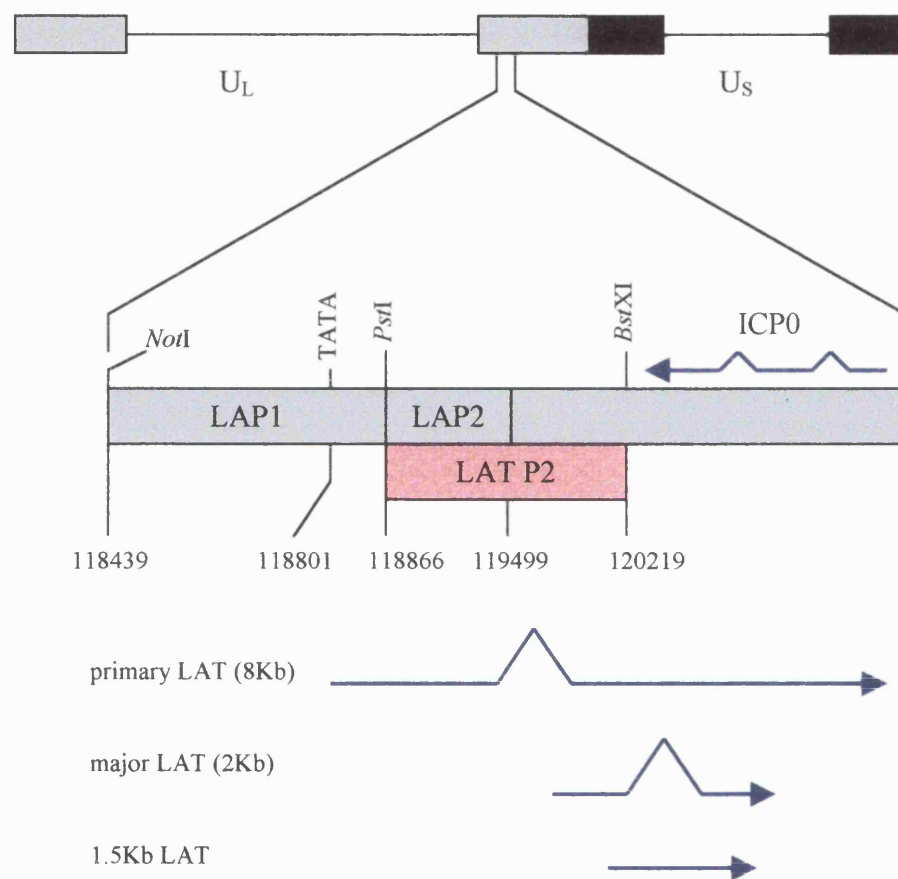
An extrapolation of this hypothesis would be to try and use LAP2 and/or surrounding regions to confer long term activity on neighbouring heterologous promoters by keeping the local DNA region transcriptionally active during latency. Theoretically, this system could be placed anywhere in the genome and would have the advantage of strong transgene expression from the heterologous promoters and long term expression afforded by the presence of the sequences derived from the LAT region. This is the basis of the design of the constructs described in this chapter.

The LAP2 promoter is defined as the 633 base pair region of the HSV1 genome immediately 5' of the stable 2kb LAT (*Pst*I-*Bam*HI, HSV1 strain KOS nt 118866-119499) (Goins *et al.*, 1994). A number of groups including our own have chosen to utilise a longer fragment of DNA (including this LAP2 promoter) in attempts to drive

long term transgene expression from HSV1 vectors. The 1.35kb fragment of DNA from the LAT region which is used in this thesis extends from the *Pst*I site at HSV1 nt 118866 to the *Bst*XI site at HSV1 nt 120219. This region of DNA is here called LAT P2 and it is similar to a region which has since been successfully used to confer long term expression on the LAP1 promoter from the locus of glycoproteinC (Lokensgard *et al.*, 1997; Berthomme *et al.*, 2000). Furthermore, inserting an internal ribosome entry site (IRES) driven transgene approximately 80 base pairs downstream of LAT P2 within the endogenous LAT region has also been found to allow long term expression to occur (Lachmann and Efstathiou, 1997). The LAT P2 region and its relationship to the rest of the HSV1 genome is shown in figure 3.1.1

The bicistronic expression cassettes described in this chapter utilise a single centrally located LAT P2 element flanked by two promoters facing away from it in a back to back orientation. Two exogenous genes can then be linked to these promoters, potentially resulting in the long term high level expression of both genes. The basic design of this cassette is shown in figure 3.1.2. A further potential advantage of this system would be the capacity for the simultaneous expression of multiple exogenous genes. This is likely to be an important consideration in a number of research and therapeutic applications and is particularly relevant to the overall aim of this project, which was to study potential interactions between wild-type and mutant APP and PS1 in the pathogenesis of Alzheimer's disease.

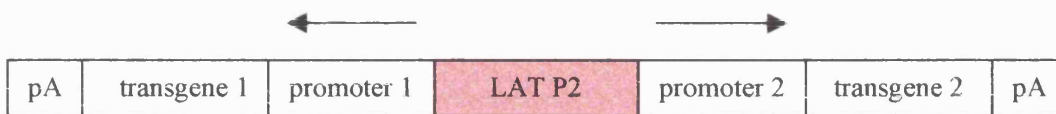
This chapter describes the construction of a number of recombinant viruses containing these bicistronic expression cassettes. The aim of the chapter was to test numerous different promoter combinations for their ability to drive high level, long term expression of two reporter genes, with a view to identifying an optimal combination of promoters on which to base future work. At the time that the work described in this chapter was carried out, it appeared that viruses disabled by the deletion of ICP27 or ICP27 together with a disabling mutation in VP16 and deletion of ICP34.5 would provide an appropriate basis for delivering genes relevant to Alzheimer's disease. These viruses were therefore used as a starting point for this thesis.



**Figure 3.1.1 The LAT P2 region**

The figure shows the LAT P2 region and its relationship to the rest of the HSV1 genome. Significant restriction sites and their positions in nucleotide base pairs (from GenBank file He1cg) are indicated. The ICP0 and LAT transcripts are shown in blue.





**Figure 3.1.2 The bicistronic expression cassette**

The expression cassette is designed such that two promoters are arranged in a back-to-back orientation with the LAT P2 element between them. The rationale behind this design is detailed in section 3.1. Briefly, it was anticipated that the altered local DNA structure surrounding the LAT P2 element would prevent transcriptional shut off of the neighbouring promoters at the onset of latency.

### 3.2 Virus nomenclature

All viruses were derived from HSV1 strain 17syn+ (Brown *et al.*, 1973). The term 1764 is used here to describe a virus with the *in1814* mutation in the gene encoding VP16 (Ace *et al.*, 1989) and with the genes encoding the neurovirulence factor ICP34.5 and ORF P completely deleted (MacLean *et al.*, 1991). The term 27- refers to the deletion of nucleotides 113273-116869, which contain the genes UL54, 55 and 56. (Howard *et al.*, 1998). UL54 is the gene encoding the essential IE gene ICP27 and UL55 and 56 are both non-essential genes. This nomenclature is summarised in table VIII. The viruses used as the starting point for this thesis were 17+27- and 1764 27-. Since both viruses are deficient in an essential gene (ICP27) they must be grown on cells which provide this deleted gene product in *trans*. Viruses 17+27-, 1764 27- and the complementing cell line (B130/2) used for their propagation had been previously constructed in the laboratory (Coffin *et al.*, 1996; Howard *et al.*, 1998).

**Table VIII: Virus nomenclature**

<b>VIRUS NAME</b>	<b>DESCRIPTION</b>
17+	HSV1 17+syn (Glasgow strain)
17+ 27-	As 17+ but with the genes encoding ICP27, UL55 and UL56 deleted
1764	As 17+ but with an inactivating mutation in VP16 and both copies of ICP34.5 and ORF P deleted
1764 27-	As 1764 but with the genes encoding ICP27, UL55 and UL56 deleted

### 3.3 Bicistronic expression cassettes

The expression cassettes used in this chapter had been previously constructed in the laboratory. The cassettes are named pR20.1 to 20.9 and are based on the structure previously described with the central LAT P2 element between two promoters arranged in a back to back orientation. The cassettes are shown in figure 3.3.1 and discussed below.

pR20.1 and 20.2 each have one LAP1 promoter and one CMV promoter. Since these cassettes were constructed, it has been shown that a region very similar to LAT P2 (LTE, Lokensgard *et al.*, 1997) is able to confer long term expression on a LAP1 promoter located downstream and facing in the opposite direction to LAP2 (virus strain -863r, (Lokensgard *et al.*, 1997). This is basically an identical construction to half of pR20.1 and pR20.2. Previous work in our laboratory had shown that a virus containing a CMV *lacZ* cassette inserted directly after LAP1 and LAT P2 in the endogenous LAT region (between the two *Bst*XI sites at nucleotides 120219 and 120406) was capable of driving long term expression in the PNS. The pair of constructs 20.1 and 20.2 were therefore a rearrangement of this organisation but were designed to allow the expression of multiple genes from sites outside of the LAT region.

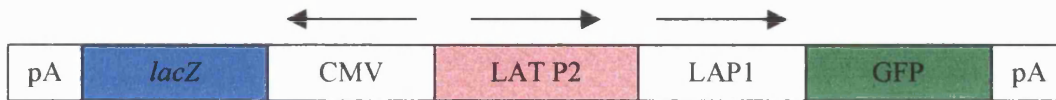
pR20.7 and 20.5 contain two heterologous promoters (two CMV promoters or one RSV and one CMV promoter respectively). The design of these cassettes is based on the hypothesis that the LAT P2 element alone is sufficient to confer long term activity on these strong promoters.

pR20.6 and 20.9 utilise the MMLV LTR promoter. It had previously been shown that this promoter either alone in ICP4 (Dobson *et al.*, 1990) or when fused to the downstream region of LAP1 in glycoprotein C (Lokensgard *et al.*, 1994) could drive long term expression. It has also been reported that the MMLV LTR promoter directly upstream of and in the opposite direction to LAP1 within the endogenous LAT region can drive transgene expression for at least 18 months post infection, albeit in a very small number of cells (Carpenter and Stevens, 1996). Thus it appears that the MMLV LTR promoter may have structural characteristics which allow it to functionally substitute for the long term expression capabilities of LAT P2. It is also a stronger promoter than either of the LAT promoters and might therefore be preferable for use in an expression cassette.

### 20.1



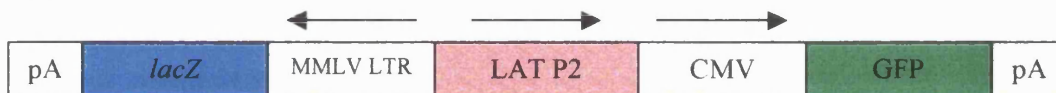
### 20.2



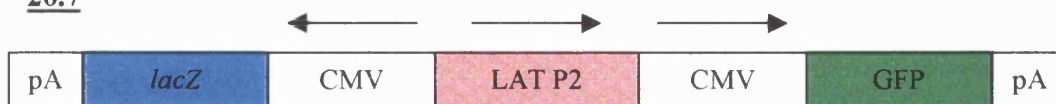
### 20.5



### 20.6



### 20.7



### 20.9



**Figure 3.3.1 The pR20 series of bicistronic expression cassettes**

The expression cassettes are all designed with two promoters arranged in a back-to-back orientation with the LAT P2 element between them. All cassettes were constructed in pGEM5. pR20.1 to pR20.9 each have a different combination of promoters as shown in the diagram. The rationale behind the choice of promoters is discussed in section 3.3.

### 3.4 The choice of ICP27 as an insertion site

ICP27 is an essential regulatory IE gene (Sacks *et al.*, 1985) encoding a nuclear phosphoprotein which performs a number of diverse regulatory functions. These include repression of IE and E genes, activation of late genes and selection of transcriptional termination sites (McLauchlan *et al.*, 1989; McCarthy *et al.*, 1989). ICP27 normally mediates the shut off of host protein synthesis by sequestering snRNPs and therefore directly inhibiting splicing (Hardy and Sandri-Goldin, 1994; Hardwicke and Sandri-Goldin, 1994). Since the majority of HSV1 transcripts are not spliced whereas the majority of cellular transcripts are, the impact of reduced splicing is much greater on host protein synthesis than on viral protein synthesis. As a result of the diversity of function of ICP27, viral mutants defective in this gene display a number of phenotypes, including a decrease in DNA synthesis, a reduction in the expression of late genes and an impairment in the shutoff of host protein synthesis (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Hardwicke and Sandri-Goldin, 1994). ICP27 is known to be toxic (Johnson *et al.*, 1994) and it has been documented that viruses deficient in ICP27 expression are of reduced cytopathogenicity compared to viruses deficient in VP16 and ICP34.5 only (Coffin *et al.*, 1996).

When constructing recombinant viruses, it is preferable to insert expression cassettes into the locus of an essential gene, such as ICP27. This ensures that if the recombinant virus undergoes homologous recombination with a wild-type virus, the resulting transfer of the expression cassette would delete the essential gene in the wild type virus, thereby providing an additional level of safety. Previous work in our laboratory had shown that viruses 17+27- and 1764 27- were efficient at gene delivery to neuronal cells both *in vitro* and *in vivo* (Howard *et al.*, 1997; Howard *et al.*, 1998).

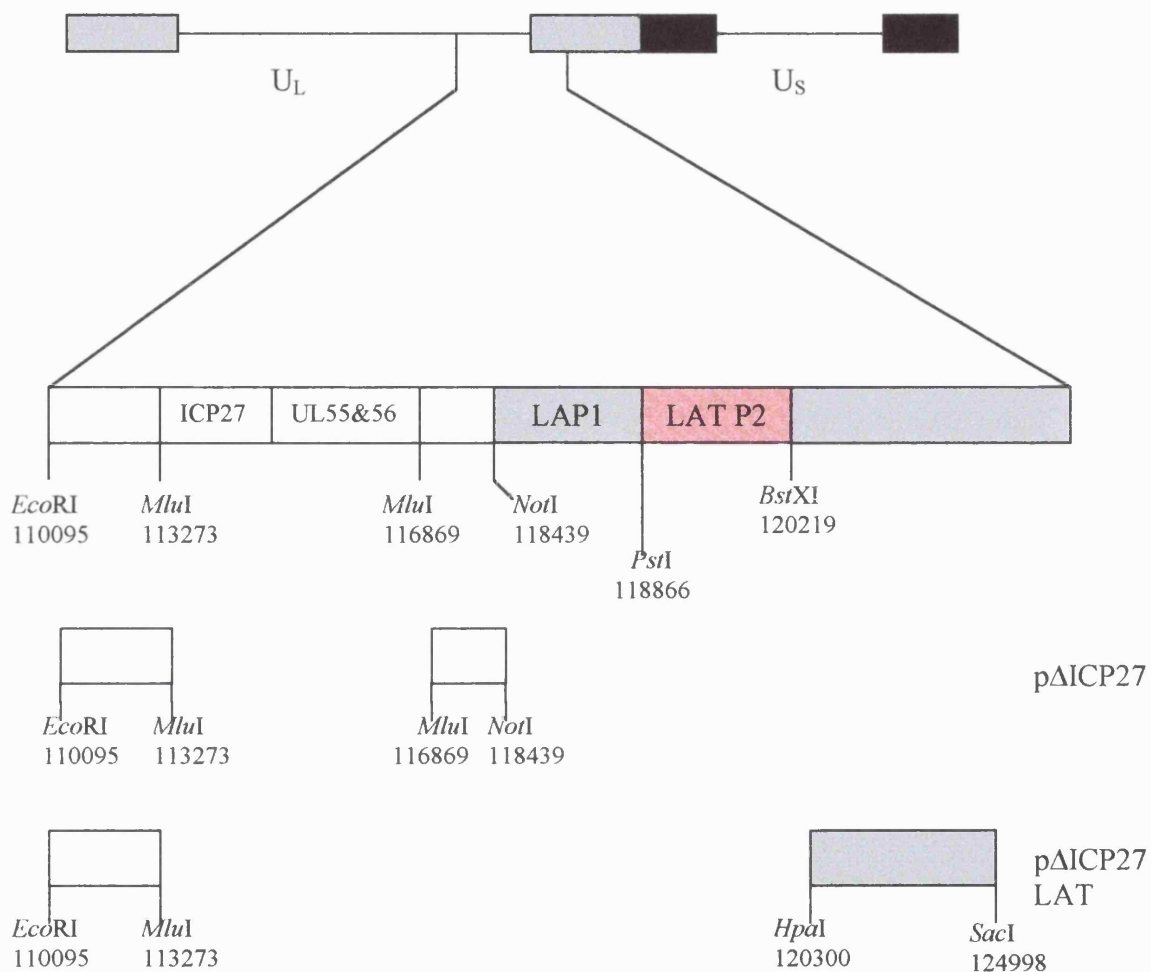
### 3.5 Insertion of expression cassettes into the ICP27 locus of 17+27- and 1764 27-

In order to facilitate homologous recombination into the ICP27 locus of the viruses 17+27- or 1764 27-, the expression cassettes must be flanked by DNA homologous to regions of the HSV1 genome outside the ICP27 gene. As described in section 3.2, the ICP27 flanking regions which had been previously used to construct viruses 17+27- and 1764 27- resulted in the deletion of the *Mlu*I fragment between nucleotides 113273 and

116869. This deleted *Mlu*I fragment contains the ICP27 gene and the non-essential genes UL55 and 56. However, if these same ICP27 flanking regions were used for the insertion of the bicistronic expression cassettes described, the LAT P2 from the expression cassette would be positioned only about 2kb 5' of the endogenous LAT P2 element in the long internal repeat. In order to avoid having the inserted and endogenous LAT P2 elements in such close proximity, the 3' ICP27 flanking region was extended such that LAP1 and the LAT P2 region in the long internal repeat would be deleted by the insertion of the cassette. These new ICP27 flanking regions were designed to result in the deletion of the region between the *Mlu*I site at nucleotide 113273 and the *Hpa*I site at nucleotide 120300. This deleted region would contain the ICP27 gene, the non-essential genes UL55 and 56, LAP1 and LAT P2. The extended ICP27 flanking regions were cloned into pSP72 such that they were separated by a *Bgl*III site which could be used for subsequent insertion of the expression cassettes. A schematic representation of the positions of the original (pΔICP27) and extended (pΔICP27LAT) flanking regions is shown in figure 3.5.1

Each of the expression cassettes has a *Srf*I site at each end and these unique sites were used for cloning into the *Bgl*III site of the extended ICP27 flanking regions, generating a set of shuttle plasmids.

Viral DNA from 1764 27- and 17+27- was prepared as described in section 2.4.7. Each shuttle plasmid was linearised using a unique *Xmn*I site in the ampicillin resistance gene of the plasmid backbone. The infectious viral DNA was co-transfected with each linearised shuttle plasmid and viral recombinants were identified by their ability to express the reporter genes GFP and *lacZ*. The viral recombinants were plaque purified as described in section 2.4.3.



**Figure 3.5.1 The ICP27 flanking regions**

The original (pΔICP27) and extended (pΔICP27LAT) ICP27 flanking regions are shown. The viruses 17+27- and 1764 27- had been previously generated using the original flanking regions whereas the bicistronic expression cassettes in the viruses described in this chapter were inserted into the extended flanking regions.

### **3.6 Viruses with LAT P2 containing cassettes in ICP27 cannot be stably propagated**

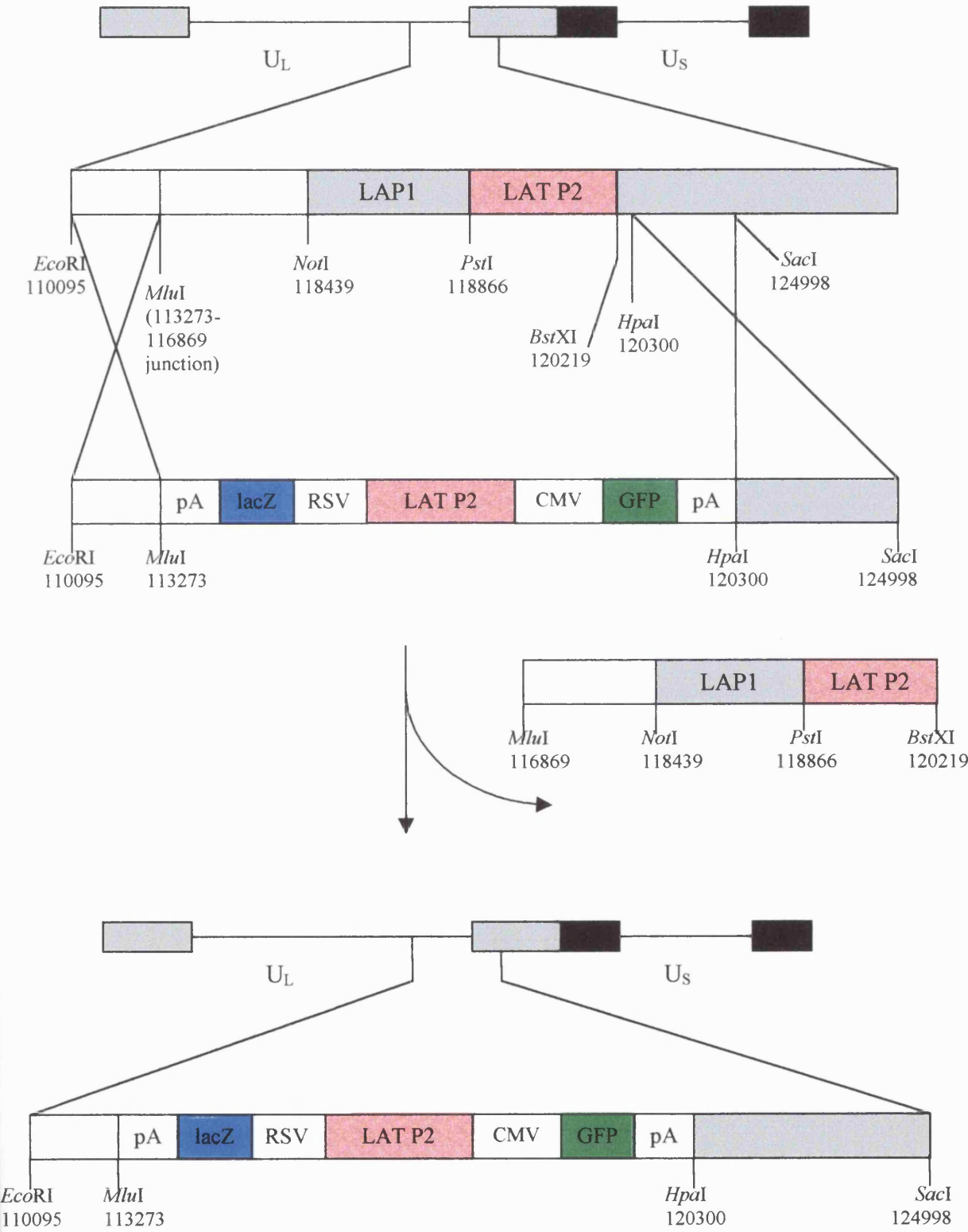
A number of viruses with expression cassettes inserted into the ICP27 locus were plaque purified and then propagated on a larger scale prior to experiments to assess promoter function. However, it became apparent at this stage that a number of the recombinant viruses were expressing either *lacZ* or GFP but not both. Consideration of the structure of the backbone viruses into which the insertions were being made revealed a possible mechanism by which at least some of these events may have occurred. This mechanism is depicted in figure 3.6.2. Briefly, it was possible that the LAT P2 region in the bicistronic expression cassette was acting as a flanking region and undergoing homologous recombination with the endogenous LAT P2 in the long internal repeat. This aberrant recombination event could have occurred either during the initial recombination event or at any time during the purification process when multiple copies of the backbone genome containing the endogenous LAT P2 regions were still present. This would result in the non-incorporation or deletion of half of the expression cassette and one of the intended flanking regions. The resulting viral recombinants would therefore only express one reporter gene.



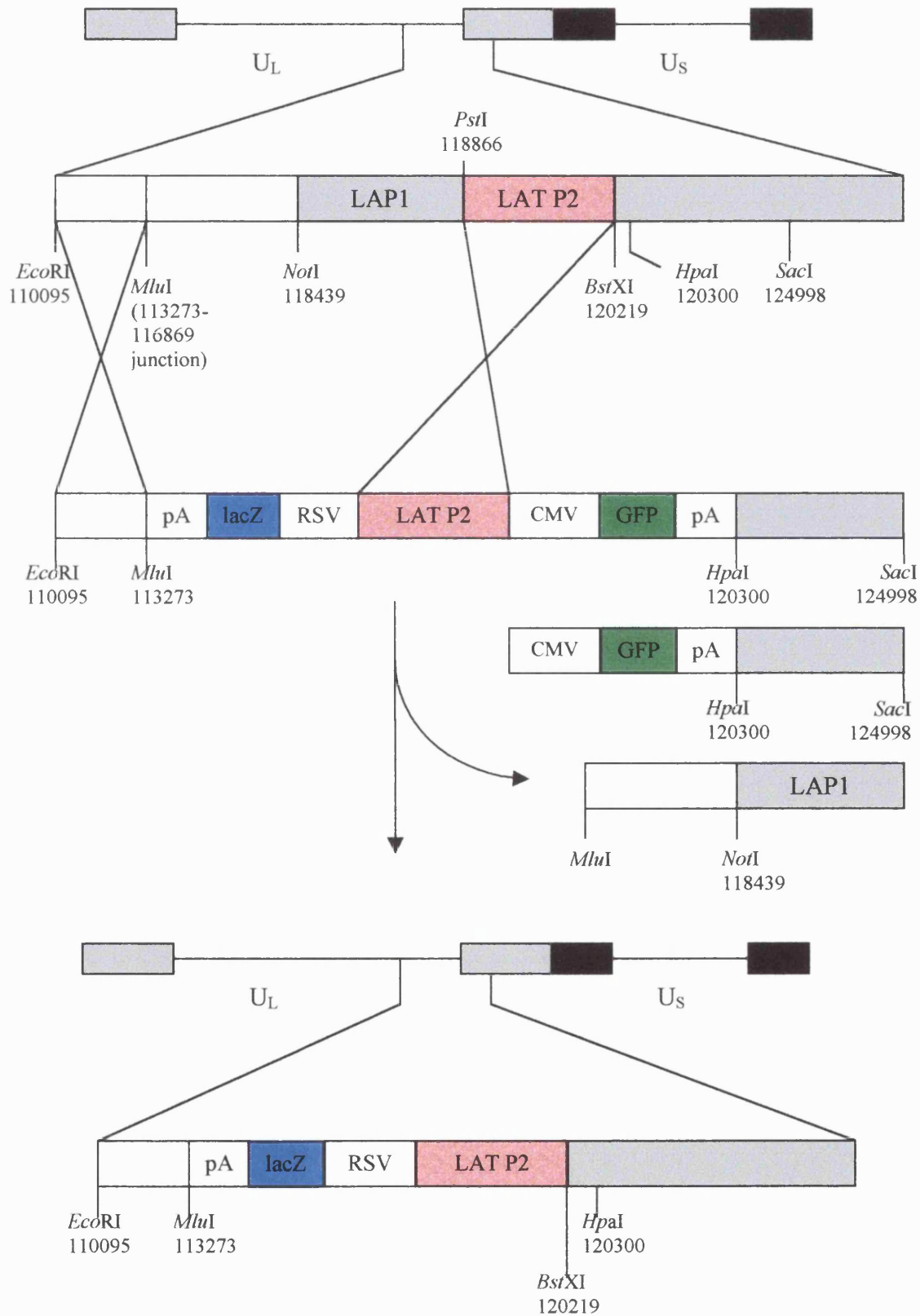
**Figure 3.6.1 Schematic diagram to illustrate the desired recombination event and a possible explanation for some of the aberrant events observed when LAT P2 containing cassettes were inserted into the ICP27 locus**

A) The predicted homologous recombination event would have resulted in the insertion of the bicistronic expression cassettes into the ICP27 locus of the viral genome in place of HSV1 nucleotides 116869 to 120219. B) The diagram depicts a possible mechanism by which some of the mutant populations which were observed may have arisen. The case of pR20.5 is used in the figure as an example but the problem was evident in the majority of viruses.

A) The predicted homologous recombination event



**B) A possible mechanism by which aberrant recombination could have occurred**



Importantly, the expression cassettes had been inserted into the extended ICP27 flanking regions in both orientations, generating two shuttle plasmids. This meant that following homologous recombination of both of these plasmids into the HSV1 genome, viruses were generated in which the inserted LAT P2 region existed in both the same and the opposite direction to the LAT P2 in the long internal repeat (which would be deleted by the insertion). These orientations are here termed forward and reverse respectively. In the viruses described above the inserts had been in the forward orientation. In an attempt to overcome the recombination problems observed, a second set of viruses were made with the expression cassettes now in the reverse orientation. It was hoped that the recombination between the inserted LAT P2 and the LAT P2 in the long internal repeat would not occur if the two elements were in opposite orientations. However, these viruses were also observed to be preferentially expressing only one of the reporter genes so their purification was abandoned. In this case, it is possible that the LAT P2 region in the inserted cassette was acting as the 5' flanking region and the *HpaI-SacI* fragment was acting as the 3' flanking region. Homologous recombination would this time be occurring with the endogenous LAT P2 in the long terminal repeat, far away from the ICP27 locus.

The viruses which were plaque purified are shown in table IX. It can be seen from the table that the aberrant recombination events described occurred in the case of all the viruses purified with the exception of 17+27- pR20.1 and 17+27-pR20.2. There were two possible explanations as to why the recombination had not taken place in the case of these viruses. Firstly, being less disabled than the 1764 27- based viruses, the 17+27- viruses were purified and propagated more easily. Additionally, the presence of LAP1 in the opposite orientation to LAT P2 in cassettes pR20.1 and pR20.2 may have in some way prevented the homologous recombination between the inserted and endogenous LAT P2 elements from taking place in such an efficient manner.

The two viruses which were stable (17+27- pR20.1 and pR20.2) were found to only drive very low levels of exogenous gene expression from the LAP1 promoter *in vitro*. These expression levels were too low to photograph. It was considered that this level of expression would not be sufficient to detect any effect resulting from subsequent insertion of the genes of interest. Parallel work in our laboratory had also

determined that viruses deleted for only ICP27 still showed toxicity to cultured neurons. The use of these two viruses for the insertion of genes relevant to Alzheimer's disease was therefore considered impractical and the approach of further developing the viruses to overcome these problems was taken.

Virus	Cassette	Orientation	Stability
17+27-	pR20.1	reverse	stable
17+27-	pR20.2	forward	stable
17+27-	pR20.5	forward	unstable
17+27-	pR20.7	forward	unstable
1764 27-	pR20.1	reverse	unstable
1764 27-	pR20.2	forward	unstable
1764 27-	pR20.2	reverse	unstable
1764 27-	pR20.5	forward	unstable
1764 27-	pR20.5	reverse	unstable
1764 27-	pR20.6	forward	unstable
1764 27-	pR20.6	reverse	unstable
1764 27-	pR20.7	forward	unstable
1764 27-	pR20.7	reverse	unstable
1764 27-	pR20.9	forward	unstable
1764 27-	pR20.9	reverse	unstable

**Table IX: Viruses with expression cassettes in ICP27 flanking regions**

The table shows the list of viruses that were purified. It can be seen from the table that the majority of viruses were unstable (i.e. were found to express either *lacZ* or GFP but not both). This observation suggested that homologous recombination between the inserted and the endogenous LAT P2 elements had occurred, deleting one of the reporter genes. This was not the case with 17+27- pR20.1 or 17+27- pR20.2, possibly for the reasons discussed in section 3.6.

### 3.7 The choice of ICP4 as an insertion site

The results discussed in section 3.6 demonstrated that ICP27 was not a suitable insertion site for the bicistronic expression cassettes containing LAT P2. This was assumed to be for two reasons. Firstly, as the ICP27 locus is close to the LAT region in the internal long repeat, homologous recombination could occur between inserted and endogenous LAT P2 regions. Secondly, the proposed aberrant homologous recombination event would result in viruses with smaller genomes which would therefore have a growth advantage over the desired recombinants. It would be preferable if the system could be designed such that any viruses resulting from undesirable recombination events would be selected against.

A more suitable insertion site might therefore be one which is further away from the LAT region and is also separated from it by a region of DNA encoding important viral functions. ICP4 is located 6.5kb away from the LAT region and the sequence between ICP4 and LAT encodes ICP0, ORF P and ICP34.5. It was anticipated that the selective pressure in favour of the ICP0 gene would be sufficient to prevent any homologous recombination between the inserted and endogenous LAT P2 elements from proving problematical.

Another major advantage of this approach would be the resulting deletion of ICP4 from the viral genome. As was discussed in the general introduction, ICP4 is the major transcriptional regulatory protein expressed by HSV1. ICP4 is absolutely required for the expression of the E and L genes and is known to be highly toxic (Johnson *et al.*, 1992). In contrast to transcripts from the other regulatory IE genes, the accumulation of ICP4 RNA was found not to be reduced as a result of the *in1814* mutation (Ace *et al.*, 1989). This suggests that the levels of ICP4 expressed by the viruses 17+27- and 1764 27- would not be reduced as a result of them containing this VP16 mutation. Additionally, an important function of ICP27 is to repress the other IE genes; thus when this repression is removed (as is the case for all the 27- viruses described in the preceding section) the remaining IE genes would be expected to be upregulated. Accordingly, the levels of ICP4 expressed from the viruses previously described in this chapter would be predicted to be high and the viruses might be

expected to be cytotoxic. It was therefore hoped that inserting the bicistronic expression cassettes into the ICP4 locus would generate viruses which were not only stable, but also non-toxic.

### **3.8 Insertion of the expression cassettes into ICP4**

The ICP4 flanking regions had been previously constructed in the laboratory from the HSV1 restriction fragment KpnE (nt 122680-134810). Briefly, A *Sau3AI* fragment (nt 123459-126774) was cloned into pSP72 (Promega) to create the 5' flanking region. A *NotI* fragment (nt 124945-125723) containing the coding region for ICP34.5 was deleted from this flanking region in order to prevent repair of the ICP34.5 deletion during homologous recombination with the virus 1764 27-. To create the 3' flanking region, a *SphI* to *KpnI* fragment (nt131730-134792) was cloned into pSP72. A *PstI*, *XbaI* and a *SalI* site from the pSP72 polylinker were left between the 5' and 3' flanking regions and these sites were used for the insertion of the bicistronic expression cassettes. All the expression cassettes shown in figure 3.3.1 were subcloned into the ICP4 flanking regions, linearised using a unique *XmnI* site in the plasmid backbone and cotransfected with infectious viral DNA. Recombinant viruses were then identified by their ability to express *lacZ* and GFP and purified as described in section 2.4.3.

Since another essential gene would be deleted by this recombination, the resulting viruses must be propagated on a cell line expressing both ICP27 and ICP4. Such a complementing cell line (B4/27) had previously been made in the laboratory. Briefly, this cell line had been generated as a result of a three-way transfection of plasmids containing ICP27 under the control of its own promoter and pA (nt 113322-115743, *BamHI-SacI*), ICP4 under the control of its own promoter and pA (HSV1 nt 126764-131730, *DdeI-SphI*) and pMAMneo (Clontech). However, as will be discussed in the next section, it became apparent that this cell line was inefficient for the growth of viruses deleted for ICP27 and ICP4.

### **3.9 Growth of viruses with ICP4 deleted is not adequately supported by cell line B4/27**

Table X shows the viruses for which purification was attempted using the B4/27 cell line. Viruses were plaque purified every two days for approximately 4 months. It can be seen from the table that the majority of viruses did not reach purity within this time. It was later found that the cell line B4/27 was unable to adequately support the growth of a virus deleted for both ICP27 and ICP4. The result of this was that the desired recombinant viruses (ICP27 deleted and ICP4 deleted) had a significant growth disadvantage as compared to the backbone viruses (ICP27 deleted only). This meant that the recombinant viruses were only readily propagated in the presence of the backbone virus, the latter providing the ICP4 required for growth. Ultimately, only one ICP27 and ICP4 deleted virus (1764 27- 4- pR20.5) was purified using this cell line.

With hindsight, the attempted generation of this number of viruses in parallel (both in this section and in section 3.6) should probably not have been carried out until the conditions for viral growth had been optimised.



<b>Virus</b>	<b>Cassette</b>	<b>Orientation</b>	<b>Purity</b>
17+27-4-	pR20.5	forward	impure
17+27-4-	pR20.7	forward	impure
1764 27-4-	pR20.5	forward	pure
1764 27-4-	pR20.5	reverse	impure
1764 27-4-	pR20.6	forward	impure
1764 27-4-	pR20.6	reverse	impure
1764 27-4-	pR20.7	forward	impure
1764 27-4-	pR20.7	reverse	impure
1764 27-4-	pR20.9	forward	impure
1764 27-4-	pR20.9	reverse	impure

**Table X: Attempted purification of viruses with inserts in ICP4**

The table shows the list of ICP4 deleted viruses for which purification was attempted using the B4/27 cell line. It can be seen that in the majority of cases the viruses were not successfully purified.

### **3.10 Viruses with LAT P2 containing cassettes inserted into the ICP4 locus cannot be stably propagated**

Soon after purification of the 1764 27- 4- pR20.5 virus had been achieved, it became apparent that this virus was unstable and undergoing genome rearrangements as had been the problem when the various cassettes were inserted into the ICP27 locus. This was somewhat surprising as the proposed mechanism by which these rearrangements were occurring would in this case have resulted in the deletion of ICP0, which would be expected to significantly impair the growth characteristics of the resulting virus.

Interestingly, the homologous recombination between the inserted and the endogenous LAT P2 regions had again occurred regardless of the orientation of the inserted LAT P2 with respect to the endogenous LAT P2. This was assumed to be either

a result of a three dimensional contortion of the viral genome or recombination in *trans* between different replicating viral genomes.

The genome instability described here may explain a previous observation by Lokensgard *et al.* in 1997. Here the authors attempted to insert a LAT P2-like element at the 5' end of LAP1 in the gC locus. They report the construction of such a virus when LAP1 and LAP2 are running in opposite directions to each other but state that they were unable to obtain an equivalent virus which contained LAP2 in the forward direction. The authors speculated that a third copy of LAP1 and LATP2 in the genome might be in some way undesirable.

Due to these instability problems, the virus 1764 27-4- pR20.5 had to be propagated by continual selection for both marker genes. As soon as this pressure was removed, the unstable viral genomes underwent rearrangements such that the gene for which active selection was not being applied was deleted. The exact recombinational mechanisms (in *cis* or in *trans*) which resulted in such an outcome have not been elucidated. Viruses resulting from such deletions had a smaller genome, giving them a growth advantage over the desired recombinants. This advantage was then amplified on scale-up such that after three to five serial passages none of the recombinant viruses had the desired characteristics of expressing both GFP and *lacZ* and the predominant population expressed neither. This meant that the 1764 27-4- pR20.5 virus could not be readily grown up in any quantity and stocks had to be prepared by the pooling of a large number of individually picked plaques. In order to confirm that the white mutant population was not a result of the virus regaining the ICP4 gene from the B4/27 cell line, this population was plated on cells expressing ICP27 only. No growth of the mutant virus was supported by the ICP27-complementing cell line, indicating that the virus had not regained the ICP4 gene.

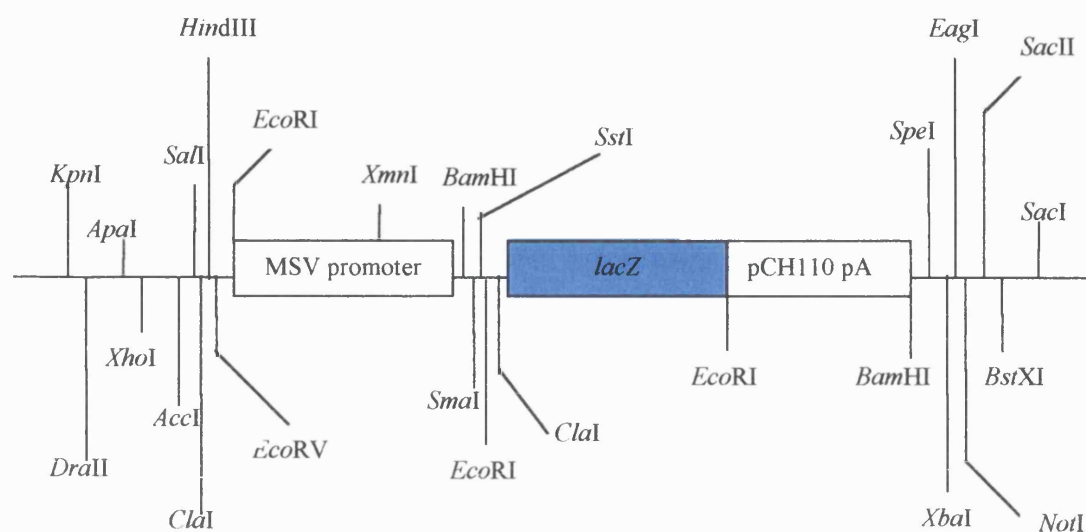
Although unlikely, it is possible that the problems encountered were not due to homologous recombination between the two LAT P2 elements, but rather a pressure not to have three copies of the LAT P2 region in the viral genome, as was suggested by Lokensgard *et al.* in 1997 (Lokensgard *et al.*, 1997). This pressure could be attributable to the proposed structure of LAT P2 which was discussed in section 3.1. Multiple copies of such an unusually structured element might, for example, destabilise the viral

genome. This instability could then lead to the deletion of either marker gene plus LAT P2 or the entire expression cassette, resulting in populations which were either blue only, green only or white. Consistent with this hypothesis, all these mutant populations were observed to exist. However, many results from ours and other laboratories have indicated that it is possible to generate less disabled viruses with multiple copies of the LAT region and that such viruses can be stably propagated (for example viruses 17+27-pR 20.1 and 20.2 described in section 3.6). Although it is possible that any selection pressure which may be associated with multiple copies of LAT P2 is slight and therefore only evident in more disabled viruses, the most likely explanation for the observed instability is aberrant homologous recombination between inserted and endogenous LAT P2 regions. It would be surprising if the advantage conferred by having a smaller genome outweighed the growth impairment resulting from the loss of ICP0, but it appears that this may have been the case with the viruses described in this section. However, whether ICP0 had in fact been deleted was not examined.

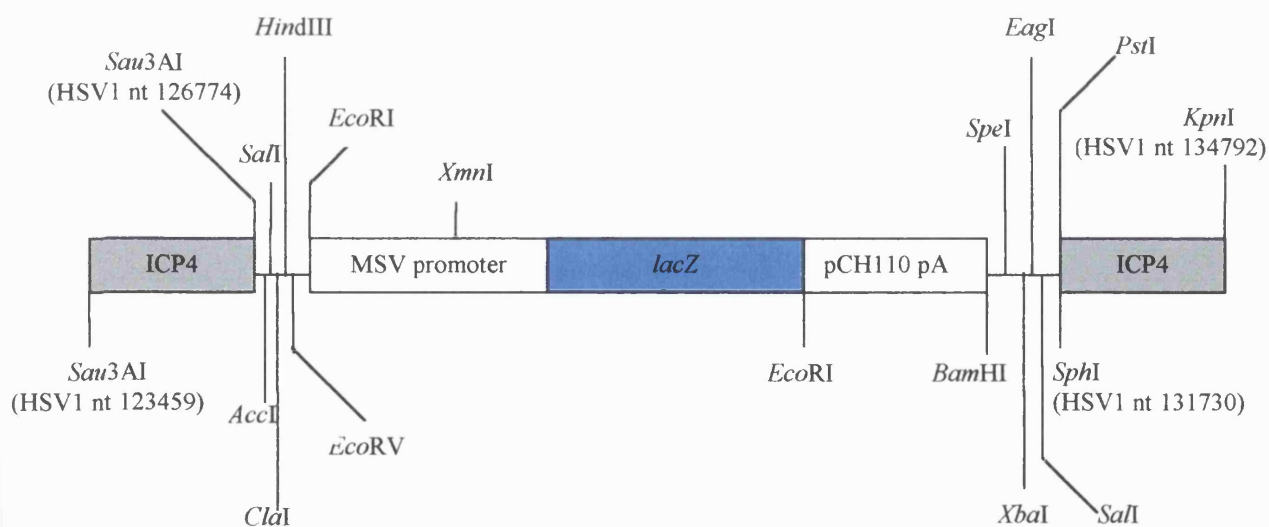
### **3.11 Attempted purification of a 1764 27- 4- virus without a LAT P2 containing cassette**

It had become apparent that deletion of ICP4 was imperative for the generation of a non-toxic virus, but that both the ICP27 and the ICP4 loci were inappropriate insertion sites for the bicistronic expression cassettes. It was therefore decided to construct a virus deleted for ICP27 and ICP4 which did not contain any inserted LAT P2 elements, thus generating a stable virus which could be used as a basis for future work. The plasmid pBSMSV-*lacZ* had been previously constructed in the laboratory and is shown in figure 3.11.1A. The MSV/*lacZ* cassette was excised from pBSMSV-*lacZ* as a *XhoI*-*NotI* fragment and inserted into the unique *XbaI* site in the ICP4 flanking regions (see section 3.8). This generated the shuttle plasmid pMSV/*lacZ*/4. A map of this shuttle plasmid is shown in figure 3.11.1B. pMSV/*lacZ*/4 was linearised using a unique *SspI* site in the plasmid backbone and cotransfected with 1764 27- viral DNA. However, despite plaque purification for several months, the inefficiency of cell line B4/27 as discussed in section 3.9 prevented this virus from being purified at this stage.

**A) pBSMSV-*lacZ***



**B) pMSV/*lacZ*/4**



**Figure 3.11.1 Map of plasmids pBSMSV-*lacZ* and pMSV/*lacZ*/4**

A) A map of the MSV-*lacZ* cassette in the cloning vector pBluescript (pBS) is shown.  
 B) A map of the shuttle plasmid pMSV/*lacZ* in ICP4 flanking regions is shown. The details of the cloning can be found in section 3.11. The plasmid can be linearised by using a unique *SspI* site in the plasmid backbone.

### 3.12 Discussion

This chapter describes bicistronic expression cassettes containing a central LAT P2 element between two promoters arranged in a back to back orientation. Attempts to incorporate these expression cassettes into a number of different viral backbones were discussed. The aims of this chapter were to generate a set of recombinant viruses containing different combinations of promoters in order to identify an arrangement which facilitated high level, long term expression of two transgenes. In this chapter, these aims were not met for the following reasons:

- 1) Insertion of the bicistronic expression cassettes into the ICP27 locus generated viruses which were unstable. This was assumed to be due to homologous recombination between inserted and endogenous LAT P2 regions.
- 2) In the viruses that were stable, the LAP1 promoter afforded only very low level expression of the transgene. It was assumed that this low level of expression would not be sufficient to detect any effect resulting from subsequent insertion of the genes of interest.
- 3) Viruses 17+27- and 1764 27- were considered to be too toxic for any useful long term expression experiments to be carried out in either cultured neurons or the CNS *in vivo*.
- 4) Deletion of ICP4 to reduce toxicity generated recombinant viruses which could not be readily purified on the currently available complementing cell line.
- 5) Insertion of the bicistronic expression cassettes into the ICP4 locus also generated viruses which were unstable.

It had therefore become apparent that for minimal toxicity, a stable ICP4- virus was necessary but the cell line B4/27 would not allow effective propagation of such viruses. This forced the use of high multiplicities of infection which may have compounded the instability observed. The main problem was considered to be that when cell line B4/27 was constructed there was no means of screening cell lines for the ability to grow a virus deleted for both ICP27 and ICP4 as such a virus did not exist in our laboratory at

the time. Therefore, due to the problems described above, all the viruses described in this chapter were abandoned with the exception of the one pure ICP4 deleted virus, 1764 27- 4- pR20.5. It was decided to generate new complementing cell lines using this virus to screen colonies for effective growth. These new cell lines would then be used to generate stable versions of the fully disabled viruses with the endogenous LAT regions deleted (see chapter 5). The construction of the new complementing cell lines is described in the next chapter.

## **CHAPTER 4**

### **CONSTRUCTION OF CELL LINES**

## 4.1 Introduction

The genome of HSV1 encodes at least 84 genes. These can be categorised as essential or non-essential genes according to whether or not they are absolutely required for the production of infectious viral particles in permissive cells. Non-essential genes provide accessory functions which contribute to pathogenicity *in vivo* but are dispensable for growth in cell culture. Essential genes are absolutely required both for growth in culture and for pathogenicity. Recombinant HSV1 vectors which have deletions in essential genes must therefore be propagated on cell lines which provide the deleted gene products *in trans*.

It has become apparent that in order to achieve minimal toxicity to target cells, a combination of both essential and non-essential genes must be deleted from HSV1. As discussed in the general introduction, studies by several groups have determined that the products of all the regulatory IE genes are cytotoxic and that in an ideal vector the expression of all IE genes would be minimised (Johnson *et al.*, 1992; Johnson *et al.*, 1994; Wu *et al.*, 1996; Samaniego *et al.*, 1998; Krisky *et al.*, 1998b). Of the IE genes, ICP27 and ICP4 are absolutely required for replication (DeLuca *et al.*, 1985; Sacks *et al.*, 1985) so deletions in these genes must be complemented for in culture. The other regulatory IE genes, ICP22 and ICP0, are classified as non-essential but their deletion significantly reduces the virus titre achievable, especially at low MOIs (Sears *et al.*, 1985; Sacks and Schaffer, 1987). The efficient propagation of viruses which express no IE genes therefore requires all the IE gene products to be provided by the complementing cell line. This presents a potential problem as the toxicity of the IE gene products means that cell lines expressing these genes are hard to generate (Krisky *et al.*, 1998b). This problem can be partially overcome by the using the promoters which normally control the expression of the IE genes to drive their expression in the cell line, since these are relatively inactive in the absence of viral infection. This approach has been successfully used to generate stable cell lines expressing single or multiple IE genes (DeLuca and Schaffer, 1987; Samaniego *et al.*, 1995; Samaniego *et al.*, 1997; Howard *et al.*, 1998). However, no cell line expressing all of the regulatory IE genes has been generated.



An alternative approach to prevent expression of the IE genes is not to delete the genes *per se* but to disable a level higher in the gene cascade and prevent the transactivation of the IE gene promoters immediately after infection. This can be achieved by mutating the gene encoding the virion protein VP16. VP16 has a dual function in the HSV1 lifecycle, it is an essential structural protein and also serves to transactivate IE gene promoters following virus infection (Batterson and Roizman, 1983; Pellett *et al.*, 1985). Because of this dual role, the gene encoding VP16 cannot be completely deleted from the virus but the inclusion of specific mutations in the C-terminal domain has been shown to greatly reduce the transactivating capability of the protein whilst maintaining the structural integrity of the virus (Ace *et al.*, 1989; Smiley and Duncan, 1997). While viruses containing these mutations show considerable growth defects, it has been demonstrated that these can be overcome in culture by the inclusion of 3mM hexamethylene bisacetamide in the growth media (McFarlane *et al.*, 1992).

Disabling VP16 as a means to reduce IE gene expression from an HSV1 vector has a potential advantage over deleting the genes individually as it does not require the complementation of all four of the regulatory IE genes by the cell line. Another possible advantage is that since the IE genes which have not been deleted remain in their natural position in the viral genome, they will remain under the control of the viral factors usually facilitating their regulated expression at the appropriate times in the virus lifecycle. This should allow the disabled viruses to be grown efficiently in culture.

The viruses which are described in the previous chapter are deleted for the two essential IE genes ICP4 and ICP27 and have a disabling mutation in VP16 (the *in1814* mutation, (Ace *et al.*, 1989). It was hoped that this combination of deletions would prevent expression of the remaining IE genes by the latter approach described above. A further level of disablement is provided by the deletion of ICP34.5 and ORF P. ICP34.5 is non-essential in all cell types except neurons (Chou *et al.*, 1990) and ORF P is only expressed when ICP4 is non-functional (Lagunoff and Roizman, 1994) so both of these genes can be deleted without the need for complementation.

The study in 1992 by McFarlane *et al.* which demonstrated that the deficits in IE gene transactivation associated with the *in1814* mutation could be overcome by addition of 3mM HMBA to the growth media applied only to viruses with this single mutation.

In this paper, the authors report a 500-fold increase in the titre achieved with *in1814* in the presence of 3mM HMBA. They also found that this effect was specific to the VP16 mutation as HMBA did not have the same effect on the yields of a virus deleted for ICP0. During the work described in the previous chapter, it was found that HMBA is insufficient at transactivating IE gene promoters in viruses which have IE gene deletions in combination with the *in1814* mutation, even if the deleted IE genes are otherwise efficiently complemented. This presented a problem in growing these multiply disabled viruses in culture. Unlike ICP4 and ICP27 which have been completely deleted from the virus, the *in1814* mutation is a 12 base pair linker insertion in the transactivation domain of VP16. This means that whilst ICP4 and ICP27 can be provided in *trans* by the complementing cell line, if unaltered VP16 was provided in this way, homologous recombination could occur between the viral DNA and the inserted DNA in the cell line, repairing the *in1814* mutation in the virus.

In an attempt to solve this problem, this chapter investigates the use of cell lines encoding a non-HSV1 homologue of VP16, gene 12 from equine herpes virus 1 (EHV1) (Purewal *et al.*, 1994). There is little if any sequence similarity between EHV1 gene 12 and HSV1 VP16 (see figure 4.1) so the risk of homologous recombination between the virus and the cell line should be minimal. Co-transfection experiments have shown that EHV1 gene 12 is capable of activating the HSV1 ICP0 and ICP4 promoters (Purewal *et al.*, 1994). It was therefore anticipated that EHV1 gene 12 might be able to functionally substitute for HSV1 VP16 during virus growth. In order to determine whether the product of EHV1 gene 12 can efficiently transactivate HSV1 IE gene promoters in the context of a virus with multiple IE gene disablements, a number of cell lines were made and tested for their ability to support the growth of a selection of disabled viruses. The work in sections 4.2 to 4.5 of this chapter was carried out in conjunction with Suzanne Thomas, UCL.

**Figure 4.1.1 Nucleotide sequence alignment between EHV1 gene 12 (shown in blue) and the gene encoding HSV1 VP16 (shown in black)**

The alignment shows that there is 46% sequence identity at the nucleotide level (using the University of Wisconsin GCG GAP program). Nucleotides 13505-14944 from EHV1 (Genbank accession number M86664) were aligned with HSV1 UL48, the gene encoding VP16 (Genbank accession number X03141, nucleotides 103607-105079).

13505 ATGTGCTCTTACATATTTCTTACCTTATTTGTGCTGTGCTCTGTTACCG...GCTGG 13561  
103607 .....CTACCCACCGTACTCGTCAATTCCAAGGGCATCGGTAAACATC 103649  
13562 TATTTTGAACGCGCGCCCGGCAGCTTCAATAGTTATGTTTGCTGCGCGGAAGAGAACGAT 13621  
103650 TGCTCAAACCTCGAAGTCGGCCATATCCAGAGCGCCGTAGGGGGCGGAGTCGTGGGGGGTA 103709  
13622 GACCCCTATCCCGGGAATCCGGCTATAATGACACCTGCGAGTCATGGATATGGACGGT 13681  
103710 AATCCCGGCCCCGGGGAATCCCGTCCCCCAACA...TGTCCAGATCGAAATCGTCTAG 103765  
13682 GCTGTGCGCAGCTTCGATGAGGGTATGCTCAGTGCCATCGAGTCCGTTTATTCCATTCCA 13741  
103766 CGCGTCGGCATGCGCCATGCGCCAGTCTCGCGTCTAAGTGAGCTCGTCCCCCAGGCT 103825  
13742 ACTAAAAGCGTCTGGCGCTGCCACCGCCCAAGGCGCGCCAGCCCGGCGCGCTATACCG 13801  
103826 GACATCGGTGCGGGGGCGGTGACAGTCTGCGCGTGTGTCCCGGGGAGAAAGGACAG 103885  
13802 CGGCTACAAGGCGAGCTGGGTTTTCCGGAGGGC...CAGACGCTTCTATCCGCTA 13853  
103886 GCGGGAGCGCGCCAGCCCGCCTCTTGGGGGGCGTGTGTCGCGGAGATCGAGCAGGCC 103945  
13854 TGGAGAAGTGGAAACGAAGACATGTTTTCTGCCCTACCCGGACATGTAGATCTATACACAG 13913  
103946 CTGATGGTAGACCGTAATTTGTTTTGTTACGCGCGGGCTGTACGCGTGTTCGCGCAT 104005  
13914 AAATCGCCCTGTGTGCGCCTCAGTAGACGAGGTAGTTAGAGCAGGCCCTCGATAGCCTGC 13973  
104006 GACCGCCTGAGGGGCGAGGTGCTGAAGCTGGAATACGAGTCCAACCTTGGCCGAATCAA 104065  
13974 CCACTCCAGCCACTATAGCCCGAGGTAGACTTGAACGCGCATGGCGACGAGCCCTCC 14033  
104066 CACCATAAAGTACCCAGAGGCGGGGCTGGTTGCCATGCAGGGTGGGAGGGGTGCTCAA 104125  
14034 CAGAGGTTCCGCG...CCTGGAAGACGACCTAGAAATATACGTGATATCGGCA 14083  
104126 CGGCGCCCTGGCTCCTCGTAGCGCGCTGCGCACCAGCGGGAGGTTAAGGTGCTCGCG 104185  
14084 CAGCGCTTTTACCTATCAGAGCTTCGACGCGCGAAGAGCACTACGCGAGGTTGCTTAGG 14143  
104186 AATGTGGTTTAGCTCCCGCAGCGCGGGCCTGATTGGCACTCCCGGACGGTGAGCGC 104245  
14144 GGCTATTGCGTAGCGCTATTGCACTACCTATACGGCAGCGCAAGCGGCAGCTTC...GC 14200  
104246 TCGTTGAAGCAACATGAAGGGCTGGAACAGACCGCCAACTGACGCCAGCTCTCCAGGTC 104305  
14201 GGAAGCGGCTCTGACGCATCTTTGATGCACAAGTTTAAACAGGTGGTGGCGACAGGTAC 14260  
104306 GCAACAGAGGCAGTCAAAACAGGTGCGGCGCATCATCTGCTCGGCGTACGCGCCCATAG 104365  
14261 TACCGCGAGGCGCTAACTTGGCCAGGTGCTGTACCTCCACTGTACGTATC...TGTT 14317  
104366 GATCTCGGGGTCAAAAATAGATACAAATGCAAAAACAAAACACGCGCCAGACGAGCGGT 104425  
14318 ACTAGGAGGATCCTGGCGCCTTACGCCAGCCAGGTAATCAATCAAGGTGTGTTGCTC 14377  
104426 CTCTGGTAGTACCTGTCCGCGATCGTGGCGCGCAGCATTTCTCCAGGTGCGGATGCGG 104485  
14378 TCGCTTCACTATTTTGGGGCGAGC...GCAGAAAGTTTGAGTGCCTGTTCC...ACCCGG 14432  
104486 TCGCGCATGTGCGCCTGGCGGTGCGAGTGCAGGACGCTGGCGCGCAGGTACCGGTACAG 104545  
14433 TGTGTGTTCAACCAAGGGGTAGTGATCTTGGAAAACGACCCCTAGAGTTCCACGATCTAC 14492  
104546 GGCAGAGCAGAAGTTGGCCAACAGGTTGATAGCTCTCTCCCGCGCCGTTAGCTCGGC 104605  
14493 AGCGGATAAACTATCG...CGAGCGGAGCTTGGCCTACCGTTGATTGCGCTGGTCTCATC 14551  
104606 GTGGAAGAAAACGAGAGAGCGCTTCGTAGTAGAGCCGAGGCGGTGCGGGTGGCGGAAG 104665  
14552 GAGGAAGAAAACAGCCCCCTGAGGCTGAGCCTCTGTTTTCGGAAAGCTACCCAGGACT 14611  
104666 CGTAGGGAAGGCCACGTGCGCGTGGGCGGAATGTGATTTGGGTGCGTTGCGGGAAGTA 104725  
14612 ATTGGCTTTCTGACGCACCAGAT...AAGAACCAAATGGAGGCATACTCGGAOGCGACCC 14670  
104726 CGCGTCCCCCATTCACCAATCGCTGGGCGAGCTTGATAGGAATTTTACACTCCCGGTA 104785  
14671 GCGGACCCCGCTCTTCTCTGCGGAGCACTCTACAGTAAACGGATAGGGGGAGCGCT 14730  
104786 CAGGTGCGGCTTGGTGGTAGCGCGGAAAACAGATCCTGTTCCAGGTATCG...AGCAT 104842  
14731 GTCATACGGTACAACGACGAGGCCATGATGGACCGCCCTCCCCAGCGCAGTGCTGCC 14790  
104843 GGTACATAGCGCGGGGCCCGCTAAAGCCCAAGTCTGAGGAGACGTTAAAGAGGGC 104902  
14791 AGGCGACCCAGTCCCGCTCTTACCGTGGGGGTGGGTCAAACCGCCGCAACGCTTGCTAT 14850  
104903 GCGGGGGGGACGGGCATGGGTGGGAGGGCATGAGCTGGGCTGGCTCAGGCGCCCGT 104962  
14851 TCGTCTAACCTCAGCTGCAGAGCATGGAACCGACGCGCTTGACTACTCATCAATGAC 14910  
104963 TGGTACAGCGGGGGGGCGCGGGGTGTTTTGGGACCCCGCGGGGGGGGGCGG 105022  
14911 GGGGATGAGCTCAACAGATGTTTGACATTTAA... 14944  
105023 TGGCGAAGCGCGTCCGCTTCATGTGCGCAACAGCTGTCGACCAAGAGGTCCAT 105079

#### **4.2 Construction of cell lines expressing ICP27 or ICP27 and EHV1 gene 12**

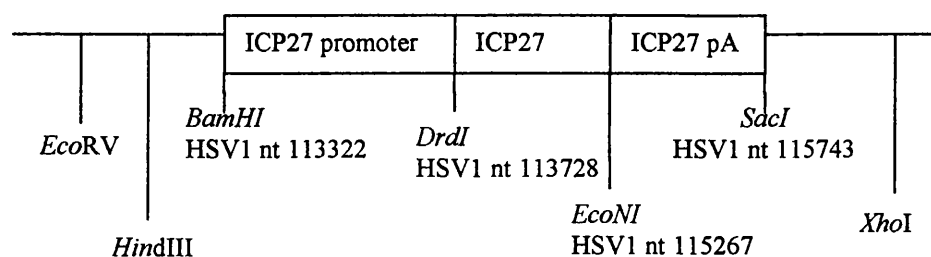
The ICP27 promoter, coding sequence and polyadenylation signal were excised from pSG130BS (Sekulovich *et al.*, 1988) as a *SacI*-*SphI* restriction fragment and subcloned into pPGKneo (Soriano *et al.*, 1991) between *EcoRI* and *SalI*. This plasmid was called p27/27/27 and is shown in figure 4.2.1A. EHV1 gene 12 inserted into pcDNA1 (pcDNA/AmpETIF) was originally provided by Matt Grapes (Marie Curie Institute, Oxted, Surrey). EHV1 gene 12 was then subcloned into pcDNA3 between the restriction sites *EcoRV* and *XbaI*. This plasmid was called pcDNA3EHV1gene12 and is shown in figure 4.2.1B. Both of these plasmids had been constructed by others in the laboratory. The plasmid 27/27/27 was either transfected on its own or co-transfected with the plasmid pcDNA3EHV1gene12 into BHK cells. Neomycin resistant colonies were selected and cell lines generated as detailed in section 2.3.7. Cell lines expressing only ICP27 or ICP27 and EHV1 gene 12 were called 27 cells or 27/12 cells respectively.

#### **4.3 Expression of EHV1 gene 12 from EHV1 gene 12 containing cells**

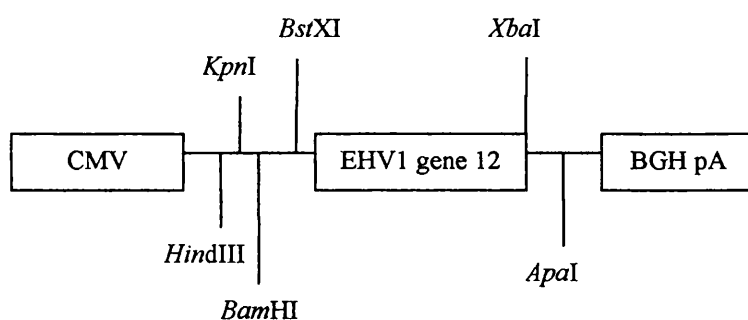
Figure 4.3.1A shows expression of EHV1 gene 12 in neomycin resistant but uncloned cells transfected with either pcDNA3 or pcDNA3 EHV1 gene 12. Figure 4.3.1B shows expression of EHV1 gene 12 in two representative cloned cell lines containing EHV1 gene 12 and ICP27 either with or without infection with a 1764 27- virus. It can be seen that in the case of the cloned cells, significant expression of the EHV1 gene 12 can only be seen in response to viral infection (although low level expression can be seen in uninfected cells on longer exposures of the blot).

This suggests that cell lines which do not constitutively express high levels of EHV1 gene12 may have a growth advantage or that expression of EHV1 gene 12 is toxic. This would mean that cell lines with low constitutive expression levels would be selected during cloning out of the cells.

### **A) p27/27/27**



### **B) pcDNA3EHV1gene12**



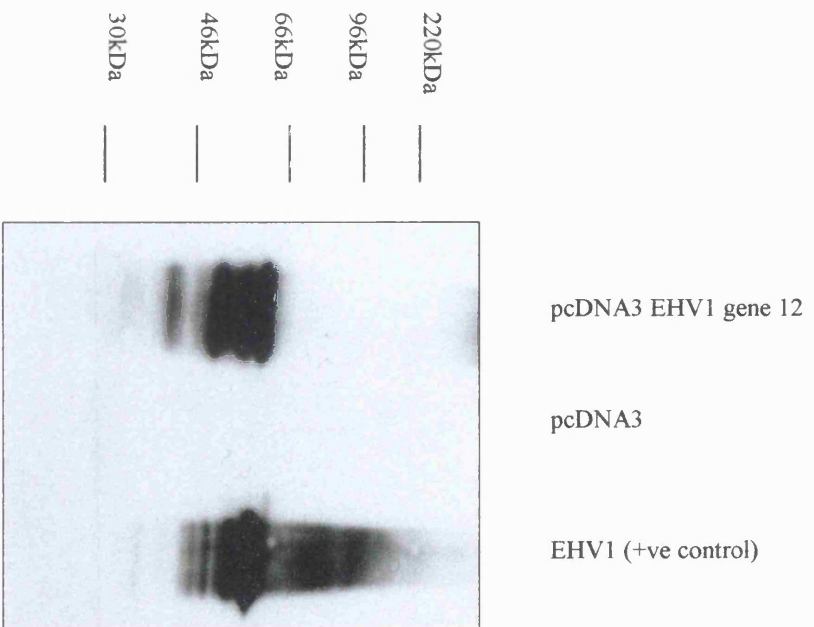
**Figure 4.2.1 Maps of plasmids p27/27/27 and pcDNA3EHV1gene12**

A) A map of p27/27/27 is shown. The backbone plasmid is pPGKneo. Details of the cloning can be found in section 4.2. B) A map of pcDNA3EHV1gene12 is shown. Details of the cloning can be found in section 4.2.

**Figure 4.3.1 Western blots showing EHV1 gene 12 expression in stably transfected BHK cells**

A) EHV1 gene 12 expression in uncloned, neomycin resistant BHK cells stably transfected with the plasmids indicated. The positive control is  $10^5$  pfu of purified EHV1 (provided by David Meredith, University of Leeds). B) EHV1 gene 12 expression in two cloned cell lines resulting from co-transfection of plasmids encoding EHV1 gene 12 and ICP27. Plus (+) or minus (-) infection refers to a 48 hour infection with a 1764 27- virus at an MOI of 5. The anti-EHV1 gene 12 antibody does not cross react with HSV1 VP16 (see figure 4.5.2).

A)



B)

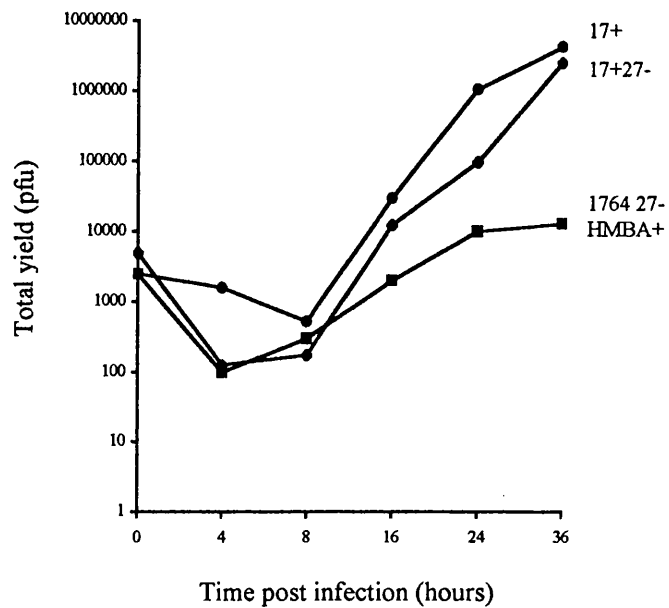




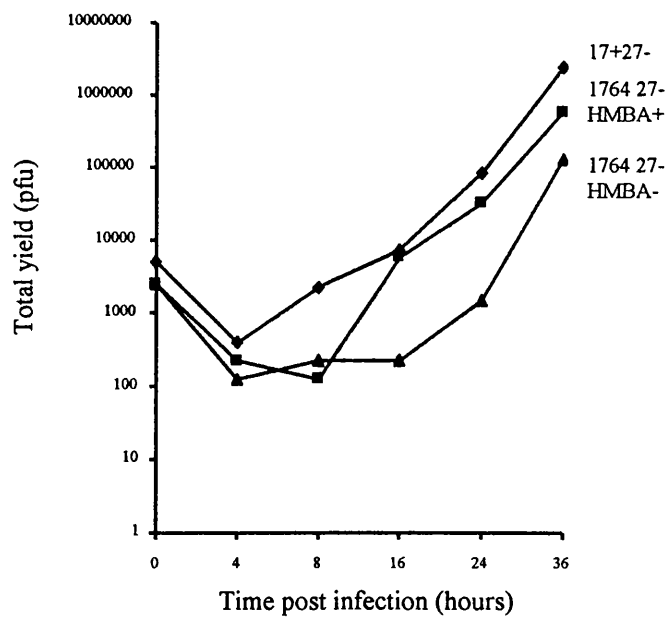
#### **4.4 Cells expressing EHV1 gene 12 and ICP27 support growth of 1764 27- viruses better than cells expressing ICP27 alone**

The cell lines described were then tested for their ability to grow disabled viruses. Figure 4.4.1 shows growth curves for a number of viruses with varying degrees of disablement on either cells expressing ICP27 only (4.4.1A) or cells expressing EHV1 gene 12 and ICP27 (4.4.1B). The growth curves were carried out at a relatively low MOI of 0.01 as it has been reported that the growth defects associated with the *in1814* mutation are more evident at low MOIs (Ace *et al.*, 1989). Figure 4.4.1A shows that on the cell line expressing ICP27 only, a 1764 27- virus gives yields over 100 times lower than a 17+27- virus over the 36 hour time course studied, even with inclusion of 3mM HMBA in the growth media. The marked difference in growth between these two viruses is likely to be attributable to the fact that the former has the *in1814* mutation whereas the latter does not (the only other difference is the deletion of ICP34.5/ORF P which has previously been shown not to significantly affect titres (MacLean *et al.*, 1991)). In contrast, figure 4.4.1B shows that when grown on cells expressing EHV1 gene 12 and ICP27, a 17+27- and a 1764 27- virus grow to approximately comparable levels.

**A) 27 cells (ICP 27 only)**



**B) 27/12 cells (ICP27 and EHV1 gene 12)**



**Figure 4.4.1 Growth curves to show growth of selected HSV1 mutants on ICP27 and EHV1 gene 12/ ICP27 containing cell lines.**

All growth curves were carried out in duplicate at an MOI of 0.01 in 24-well plates.

Yields are given as total values in pfu/well (500µl).

#### **4.5 The product of EHV1 gene 12 is not packaged into HSV1 virions**

The purpose of a complementing cell line in vector production is to enable virus propagation in culture. When the vector virus is purified away from the complementing cell line, it should retain its full level of disablement. As was shown in the alignment in figure 4.1, there is minimal sequence similarity at the DNA level between EHV1 gene 12 and HSV1 VP16 so the potential for homologous recombination should be low. However, it is important to consider the possibility that the protein product of EHV1 gene 12 could be packaged into HSV1 virions. This is a consideration since HSV1 VP16 and the product of EHV1 gene 12 are both phosphoproteins which are located in the tegument of their respective viruses. If the EHV1 gene 12 protein expressed from the cell line was packaged into HSV1 virions, functional transactivating protein would be carried over to target cells where HSV1 IE gene promoters would then be activated. This would mean the beneficial disabling effects of the *in1814* mutation would be lost.

Figure 4.5.1 shows the sequence similarity at the protein level between HSV1 VP16 and the product of EHV1 gene 12. It can be seen from this alignment that the two proteins are only 34% identical at the amino acid level. This information, combined with the observation that VP16 mutated HSV1 stocks grew identically on non-engineered BHK cells regardless of whether or not the stocks had been previously propagated in cells containing EHV1 gene 12 (Suzanne Thomas, personal communication), led us to believe that packaging was unlikely. However, the packaging signal might be very small so in order to conclusively show if the product of EHV1 gene 12 was capable of being packaged into HSV1 virions, the following experiment was carried out. Stocks of virus strains 1764 or 1764 27- were grown on cells either containing or not containing EHV1 gene 12. These virus stocks were then purified as detailed in section 2.4.4 and loaded directly onto polyacrylamide gels. Western blots were then performed using antibodies directed against HSV1 VP16 or the product of EHV1 gene 12 (antibody provided by Gretchen Caughman, Medical College of Georgia). This western blot is shown in figure 4.5.2. It can be seen that there is a strong anti-VP16 signal but no anti-EHV1 gene 12 signal for all the HSV1 viruses regardless of the cell line on which the viruses were prepared. It can therefore be concluded that

the product of EHV1 gene 12 is not packaged into HSV1 virions. This suggests that when the mutant viruses are purified away from the complementing cell line, impairments in IE gene transactivation associated with the *in1814* mutation in VP16 will be retained. This result also serves to confirm that the growth deficiencies associated with the *in1814* mutation are indeed due to deficiencies in transactivation rather than an effect on the structural role of VP16, which was previously still formally possible (Roizman and Sears, 1996).

```

1 MCLLHISLPYLSCALLPGWYFDARPAASIVMFAAAEENDDPYPGKSGYNDTC..EL...M 55
  |||||.....|.....|.....|
1 MDLL.....V.....D.....ELFADM 12

56 DMDGAV....ASFDEGMLSAIESVYSIP..TKKRLALPPPKAASPGALYQRLQGELGFP 108
  .|||.....|.....|.....|.....|.....|.....|.....|.....|
13 NADGASPPPPRPAGGPKNTPAAPPLYATGRLSQAQLMPSPMPVPPAALFNRLDDLGF 72

109 EGQTLLSAMEKWNEDMFSALPGHVDLYTEIALLSTSVDEVVRAGLDSLPTPSHYSPEVDL 168
  |...::|||:|||||...|||...|||...:|...|...:|:
73 AGPALCTMLDTWNEDLFSALPTNADLYRECKFLSTLPSDVVEWGDAYVPERT....QIDI 128

169 NAHGDEPFPEVPALEDDLEIYVISAQRFYSELRTREEHYARLLRGYCVALLHYLYGSAK 228
  |||||...|...|...|...|...|...|...|...|...|...|...|...|
129 RAHGDVAFPTLPATRDGLGLYYEALSRLFHAELRAREESYRTVLANFCSALYRYLRASVR 188

229 ...RQLRGSGSDASLMHKFKQVVRDRYYREANLARLLYLHLYVSVTREVSWRLHASQVI 285
  ||...|...|...|...|...|...|...|...|...|...|...|...|...|
189 QLHRQAHMRGRDRDLGEMLRATIADRYRETARLARVLFHLHYLFTREILWAAYAEQMM 248

286 NQGVFVSLHYFWAQRKFECLFHPVLFNHGVVILENDPLEFHDQLRINYRRRELGLPLIR 345
  .||...|...|...|...|...|...|...|...|...|...|...|...|...|
249 RPDFDCLCCDLESWRQLAGLFQPFMFVNGALTVRGVPIEARRLRELNHIREHLNPLVR 308

346 AGLIEEENSPLAEPLFSGKLPRTIGFLTHQIRTKMEAYSDAHPATPLFPLAEHSYSKRI 405
  .||...|...|...|...|...|...|...|...|...|...|...|...|...|
309 SAATEEPGAPLTPPTLHGNQARASGYFMVLIRAKLDSYSSFTTSPSEAVMREHAYS.RA 367

406 GGRLSYGTTTEAMMDPPSPSAVLPGDPVPP...LTVGVRQTAATLAIPSNLTL.QSMET 460
  :...|...|...|...|...|...|...|...|...|...|...|...|...|
368 RTKNNYGSTIEGLLDLPDDAPEEAGLAAPRLSFLPAGHTRRLST.APPTDVSLGDELHL 426

461 DGLDYSSMTGDELNQMFDI*..... 480
  ||...|...|...|...|...|...|
427 DGEDVAMAHADALDD.FDLMDLGDGDSPPGFTPHDSAPYGALDMADFEFEQMFT 480

Percent Similarity: 43.529
Percent Identity: 34.000

```

**Figure 4.5.1 Sequence similarity at the amino acid level between HSV1 VP16 (shown in black) and the product of EHV1 gene 12 (shown in blue)**

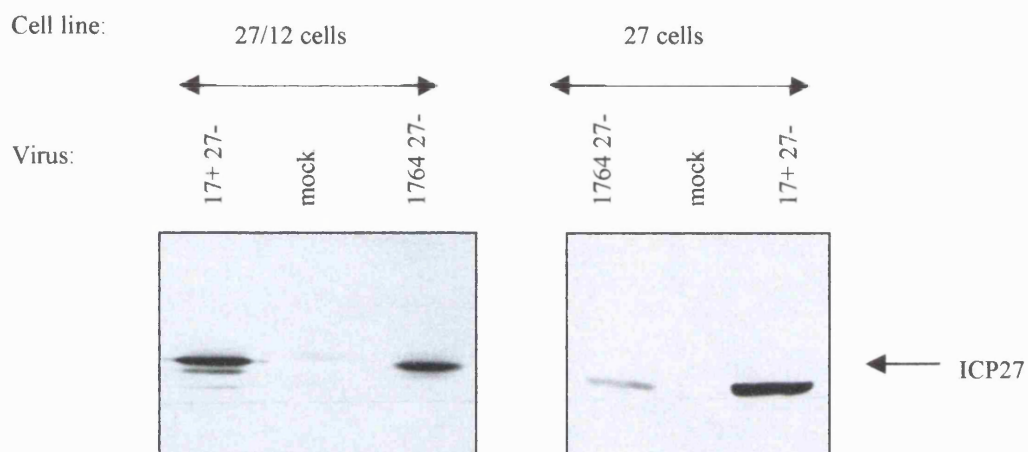
The alignment shows that there is 34% sequence identity at the amino acid level (using the University of Wisconsin GCG GAP program). EHV1 gene 12 protein (AAB02447.1) is shown in blue and HSV1 VP16 (CAA26913.1) is shown in black.



#### **4.6 ICP 27 is not constitutively expressed by the 27/12 cell line but is induced in response to viral infection**

As discussed in section 4.1, the products of all the IE genes are known to be cytotoxic. It is therefore important that the IE genes contained in the cell lines are not constitutively expressed in the presence of EHV1 gene 12. This might be expected given that the product of EHV1 gene 12 is known to transactivate HSV1 IE gene promoters (Purewal *et al.*, 1994). It was hoped that the low level expression of EHV1 gene 12 in the absence of viral infection (figure 4.3.1B) would not on its own be sufficient to transactivate the IE gene promoters inserted into the cell line genome.

Figure 4.6.1 shows a western blot of 27 and 27/12 cell samples before and after infection with an ICP27 deficient or an ICP27 and VP16 deficient virus. It is apparent from this western blot that only very low levels of ICP27 are produced from either cell line in the absence of viral infection. Cotransfection experiments in our laboratory have shown that the product of EHV1 gene 12 is able to transactivate the ICP0 and ICP4 promoters to a greater extent than the ICP27 promoter, suggesting a possible explanation for this observation (Thomas *et al.*, 1999a). Figure 4.6.1 also shows that the level of ICP27 in the 27/12 cells is greatly increased 24 hours after infection with either an ICP27 deficient or an ICP27 and VP16 deficient virus. This was not the case in the 27 cells, where expression of ICP27 was greatly increased following infection with an ICP27 deficient virus but only relatively weakly increased following infection with a virus deficient in both ICP27 and VP16. This suggests that the low level of EHV1 gene 12 constitutively expressed by cell line 27/12 is sufficient to induce the expression of an activating factor in the incoming virus which can then in turn activate other promoters such as ICP27. We propose that this incoming factor is ICP0. This possible gene regulation mechanism will be discussed further in section 4.10.



**Figure 4.6.1 Western blot to show ICP27 expression is induced in response to virus infection**

Samples from either ICP27 or ICP27 and EHV1 gene 12 containing cells were harvested 24 hours post infection with the viruses indicated at an MOI of 5. Extract from approximately  $10^5$  cells was loaded per lane. Since none of the viruses used in this experiment encode ICP27 (the gene is completely deleted in each case) all the ICP27 protein detected in the figure result from expression from the cellular DNA.

#### 4.7 Construction of cell lines expressing ICP4 under the control of the ICP27 promoter

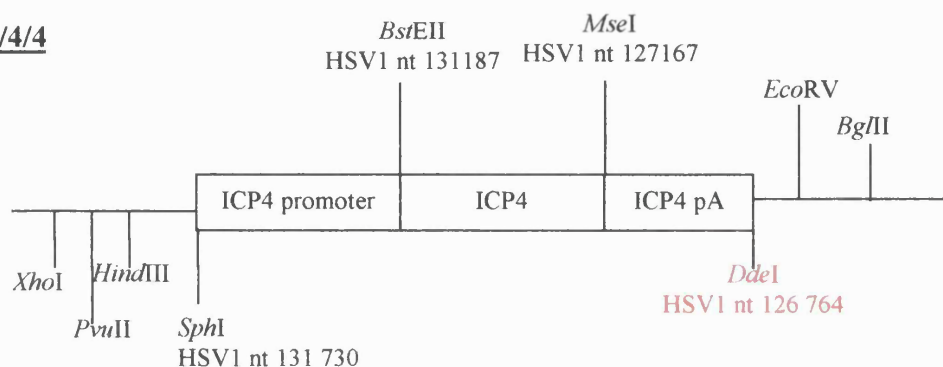
The results in the preceding section demonstrate that the ICP27 promoter is not constitutively activated by the low levels of EHV1 gene 12 continually expressed by the cell line but is efficiently induced in response to viral infection. It was therefore speculated that the ICP27 promoter might provide suitable regulation of ICP4 in a cell line designed to express ICP27, EHV1 gene 12 and ICP4. In order to test this hypothesis, a plasmid was constructed which encoded zeocin resistance and ICP4 under the control of the ICP27 promoter and polyadenylation signal. This plasmid was constructed by cloning the ICP4 promoter, coding region and polyadenylation signal excised as a *DdeI-SphI* fragment (nt 126764-131730) from the HSV1 KpnE genomic fragment (nt 122680-134810) between the *EcoRI* and *SphI* restriction sites in pSP72. This plasmid was called p4/4/4. The ICP27 pA was then excised from pSG130BS (Sekulovich *et al.*, 1988) as an *EcoNI-SacI* restriction fragment (nt 115267-115743) and inserted between the *MseI* (nt 127167) and *EcoRV* (pSP72 polylinker derived) sites in p4/4/4. This generated plasmid p4/4/27. The ICP27 promoter was then excised from pSG130BS as a *SphI-DrdI* fragment and inserted between the *SphI* and *BstEII* sites in p4/4/4 (nt 131730-131187). This generated plasmid p27/4/27. The zeocin resistance cassette was excised from pVgRXR (Invitrogen) as a *BamHI* fragment and inserted into the unique *BglII* site in p27/4/27 to create p27/4/27/zeo. The structure of p27/4/27/zeo is shown in figure 4.7.1. p27/4/27zeo was then transfected into the previously described 27/12 cells (see section 4.2). Zeocin and neomycin resistant colonies were picked and cell lines generated as described in section 2.3.7. 140 colonies were screened for their ability to grow viruses deficient in VP16, ICP27 and ICP4. The best of these was named 27/12/27:4 and selected for further investigation.

Growth curves were performed using the virus strain 1764 27- and the newly purified 1764 27- 4- virus, the construction of which was described in chapter 3. The results of these experiments are shown in figure 4.7.2. It can be seen that the cell line 27/12/27:4 is unable to support significant growth of a virus deleted for ICP4, even though it was the best clone identified out of the 140 screened. It would therefore

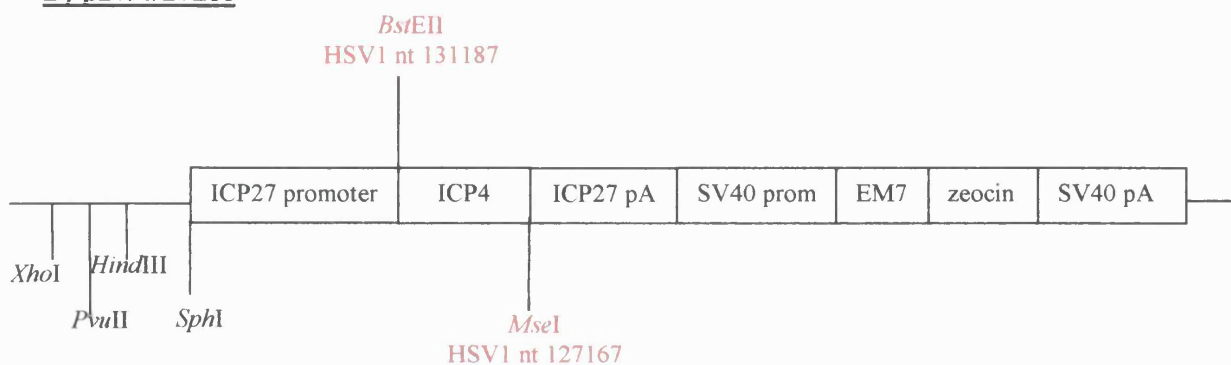


appear that whilst the ICP27 promoter is capable of directing optimal expression of ICP27 from a cell line, it is inappropriate for controlling the expression of ICP4.

### A) p4/4/4

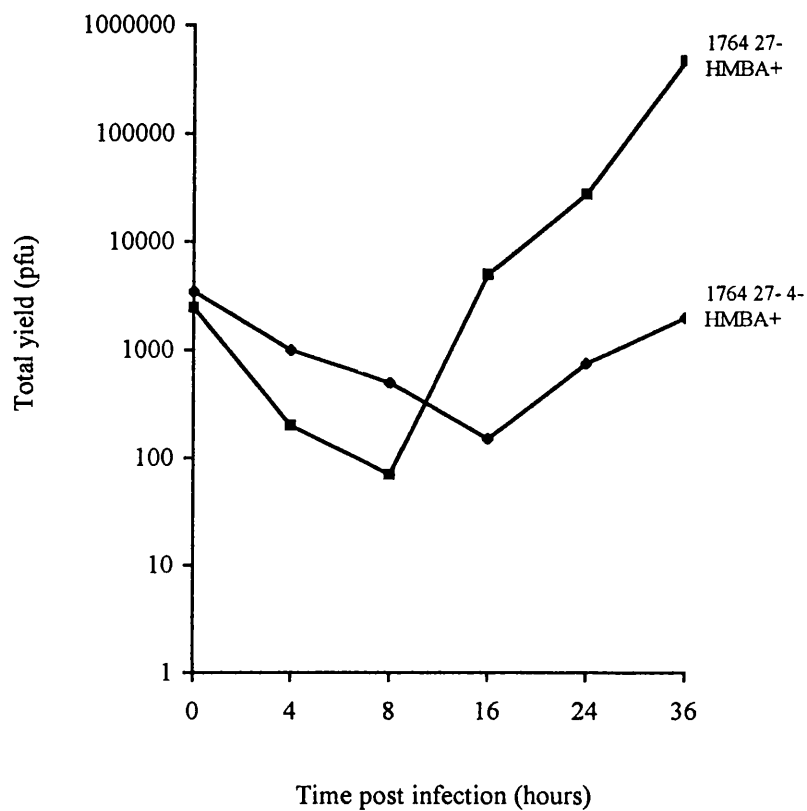


### B) p27/4/27zeo



**Figure 4.7.1 Cloning of plasmid p27/4/27zeo**

A) Map of plasmid p4/4/4. The details of the cloning can be found in section 4.7. Briefly, p4/4/4 is the ICP4 promoter, coding sequence and pA cloned into pSP72 (Promega). The *DdeI* site shown in red was destroyed in the cloning and is indicated for orientation purposes only. B) Map of plasmid p27/4/27/zeo. The details of the construction of this plasmid can be found in section 4.7. Briefly, the ICP4 pA was removed from p4/4/4 and replaced with the ICP27 pA on a *EcoNI/SacI* fragment from pSG130BS (Sekulovich *et al.*, 1988). The ICP4 promoter was removed from p4/4/4 and replaced with the ICP27 promoter as a *SphI/DrdI* fragment from pSG130BS and the zeocin resistance cassette from pVgRXR (Invitrogen) was cloned into the unique *BglII* site. The sites indicated in red have been destroyed in the cloning and are indicated for orientation purposes only.



**Figure 4.7.2 Expression of ICP4 afforded by the ICP27 promoter does not allow effective growth of 1764 27- 4- viruses**

The growth curves were carried out in duplicate at an MOI of 0.01 in 24-well plates. 3mM HMBA was included in the growth media. Yields are given as total values in pfu/well (500 $\mu$ l).

#### 4.8 Construction of cell lines expressing ICP4 under the control of the ICP4 or MMTV promoters

Since the ICP27 promoter is unable to drive expression of ICP4 appropriate for efficient virus growth, further cell lines were generated with the ICP4 coding sequence under the control of either its own promoter and polyadenylation signal or the mouse mammary tumour virus (MMTV) promoter and SV40 polyadenylation signal.

The MMTV promoter (pMAMneo, Invitrogen) is inducible by 1 $\mu$ M dexamethasone (Lee *et al.*, 1981). The rationale behind the construction of these cell lines was that either ICP4 expression would be appropriately regulated by its own promoter or alternatively, expression of ICP4 from the MMTV promoter could be artificially induced by addition of dexamethasone prior to or at the time of infection. It is known that EHV1 gene 12 can potently transactivate the HSV1 ICP4 promoter (Purewal *et al.*, 1994), and that the effect is more pronounced on this promoter than on the ICP27 promoter (Thomas *et al.*, 1999a). It was therefore a distinct possibility that the low levels of EHV1 gene 12 constitutively expressed by the 27/12 cell line would continually transactivate the ICP4 promoter, thereby generating unacceptably high background levels of the toxic ICP4 protein. The second cell line, with the inducible MMTV promoter controlling the expression of ICP4 was therefore designed in an attempt to avoid this potential problem.

The plasmids used to make these cell lines were named p4/4/4/zeo and pMAMzeo/4 respectively. Plasmid p4/4/4/zeo was constructed by cloning the zeocin encoding *Bam*HI fragment from pVgRXXR (Invitrogen) into the unique *Bg*II site in plasmid p4/4/4 (see section 4.7). The structure of plasmid p4/4/4zeo is shown in figure 4.8.1A. The plasmid pMAMzeo/ICP4 was based on the plasmid pMAMneo (Clontech). The neomycin resistance gene from pMAMneo was removed as a *Bam*HI fragment and replaced with the zeocin encoding *Bam*HI fragment from pVgRXXR (Invitrogen). This plasmid was named pMAMzeo. The ICP4 coding region (HSV1 nt 127167 to 131187, *Mse*I/*Bst*EII) was then excised from p4/4/4 (see section 4.7) and inserted into the unique *Xho*I site in pMAMzeo, creating pMAMzeo/ICP4. The structure of pMAMzeo/ICP4 is shown in figure 4.8.1B. The plasmids p4/4/4zeo and pMAMzeo/ICP4 were linearised

and separately transfected into the previously described 27/12 cells (see section 4.2). Zeocin and neomycin resistant colonies were picked and cloned out and cell lines generated as described in section 2.3.7. In the case of the ICP4 promoter driving expression of ICP4, 138 colonies were picked and screened for their ability to grow viruses deficient in VP16, ICP27 and ICP4. The best of these was named 27/12/4:4 and selected for further investigation. In the case of the MMTV promoter driving expression of ICP4, 88 colonies were picked and screened for their ability to grow viruses deficient in VP16, ICP27 and ICP4. The best of these was named 27/12/M:4 and selected for further investigation. When these colonies were undergoing screening, it became apparent that there were marked differences in the proportion of clones which were able to support growth of a virus deficient in VP16, ICP27 and ICP4. When ICP4 was controlled by its own promoter, the vast majority of clones were of only limited permissivity for the disabled virus, with only two facilitating significant productive virus growth (as assayed by the presence of significant CPE within 48 hours of infection). However, when the MMTV promoter was driving the expression of ICP4, more than half the colonies picked enabled relatively efficient propagation of the disabled virus. This observation is summarised in table XI.

Promoter driving ICP4 expression	Number of clones picked	Number of clones able to complement ICP4	% of clones able to complement ICP4
ICP4	138	2	1.4
MMTV	88	60	68

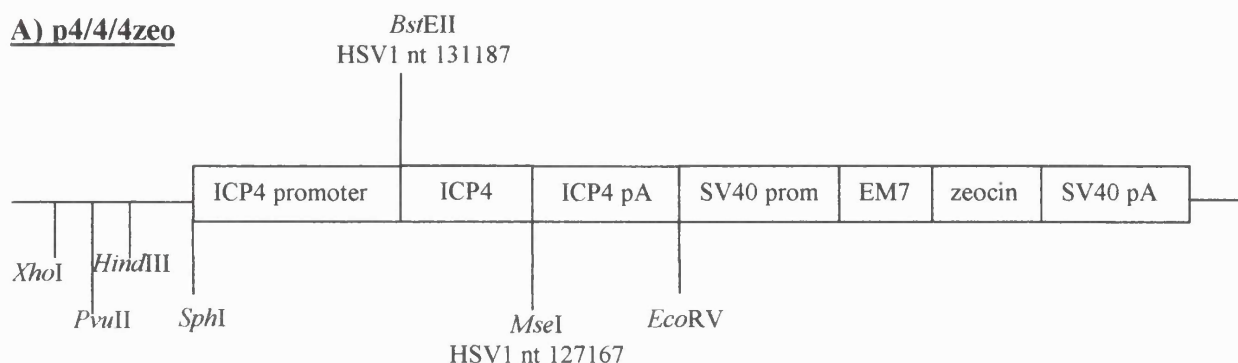
**Table XI: The effect of promoter choice on the percentage of clones capable of complementing ICP4 deficiencies in a virus lacking functional VP16, ICP27 and ICP4**

These results could be related to the integration site of the plasmid containing ICP4 under the control of its own promoter in the cellular genome. It is possible that in the case of the 27/12/4:4 cell line, the ICP4 plasmid may have been preferentially inserted

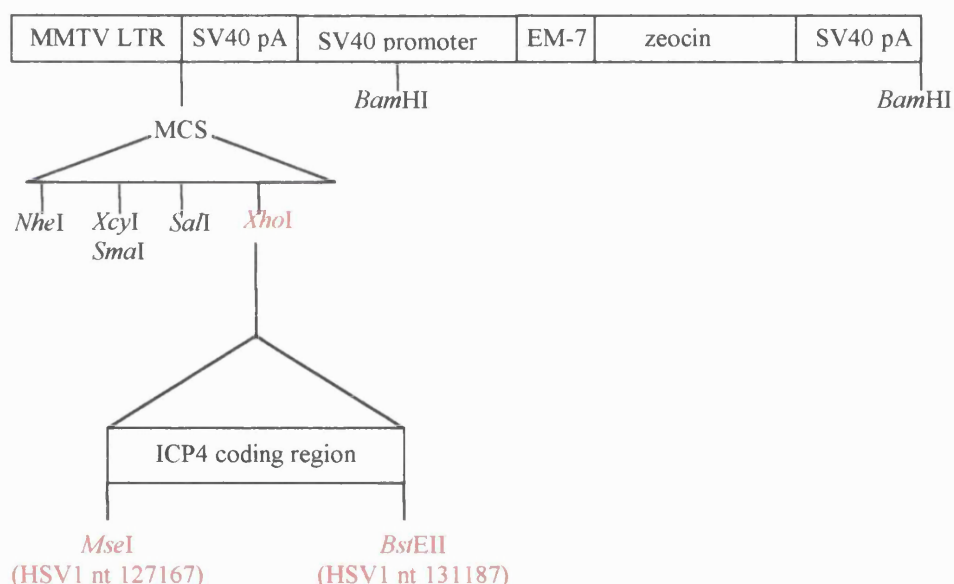
into a site which either does not allow ICP4 to be expressed or in which ICP4 is constitutively expressed. Western blotting for ICP4 levels from this cell line (results presented in figure 4.9.1) suggested that the latter explanation was more likely. Given the known responsiveness of the ICP4 promoter to EHV1 gene 12 (Purewal *et al.*, 1994), such positional effects are likely to be important in the context of a EHV1 gene 12 containing genome. It could be, for example, that in the large percentage of clones which are unable to support growth of the disabled virus, integration has occurred at sites which expose the ICP4 promoter to constitutive activation by the low levels of EHV1 gene 12 continually expressed from the cell line. High levels of toxic ICP4 would occur, selective pressure then causing these clones to lose or prevent expression from the ICP4 containing plasmid. Such positional effects seem not to be important in the case of the MMTV promoter which appears to provide appropriate regulation of ICP4 regardless of the insertion site.

Figure 4.8.2 shows growth curves for cell lines 27/12/4:4 and 27/12/M:4. It can be seen that both cell lines support growth of a virus deficient in VP16, ICP27 and ICP4 (1764 27- 4-) to nearly equivalent titres as a virus deficient in only VP16 and ICP27 (1764 27-). Figure 4.8.2 also shows that the effect of 3mM HMBA on viral growth is now negligible, demonstrating the effectiveness of EHV1 gene 12 in complementing the function of HSV1 VP16 in this system.

### A) p4/4/4zeo



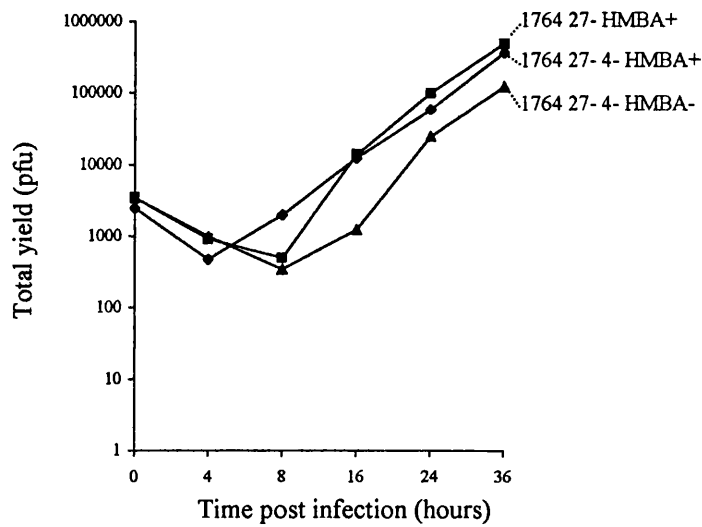
### B) pMAMzeoICP4



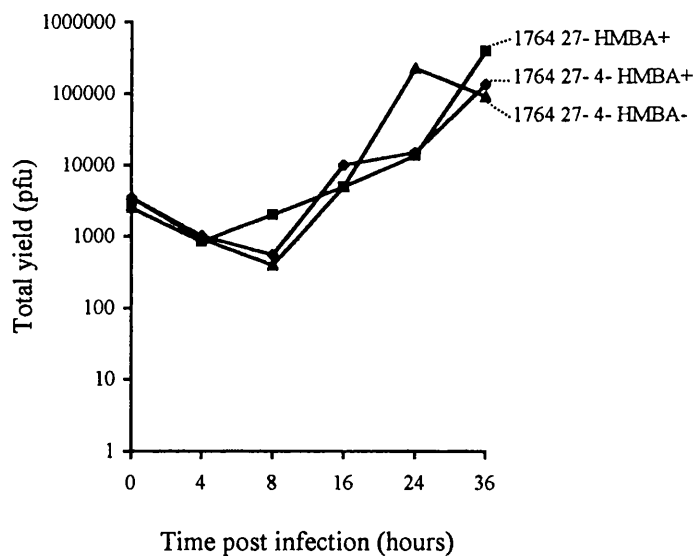
**Figure 4.8.1 Maps of plasmids p4/4/4zeo and pMAMzeoICP4**

A) Plasmid p4/4/4 was constructed from p4/4/4 (see section 4.7) by inserting a zeocin resistance cassette into the unique *BglII* site derived from the pSP72 polylinker. B) Plasmid pMAMzeoICP4 was generated from pMAMneo by replacing the neomycin resistance cassette with one encoding zeocin resistance and inserting the ICP4 coding sequence into the unique *XhoI* site. Details of the cloning strategies can be found in section 4.8. Sites indicated in red have been destroyed in the cloning and are indicated for orientation purposes only.

**A) 27/12/4:4 (ICP4 under ICP4 promoter)**



**B) 27/12/M:4 (ICP4 under MMTV promoter)**



**Figure 4.8.2 The expression of ICP4 from either the MMTV promoter or the ICP4 promoter allows effective growth of viruses deficient in VP16, ICP27 and ICP4**

Growth curves of viruses 1764 27- and 1764 27- 4- on cell lines with either A) the ICP4 promoter (cell line 27/12/4:4) or B) the MMTV promoter (cell line 27/12/M:4) are shown. All growth curves were carried out in duplicate at a MOI of 0.01 in 24-well plates. Yields are given as total values in pfu/well (500µl).



#### 4.9 The MMTV promoter and the ICP4 promoter are induced by viral infection

The responsiveness of the CMV promoter and the ICP27 promoter to infection with less disabled viruses were discussed in section 4.4 and 4.6 respectively. In order to determine if the ICP27, ICP4 and MMTV promoters were induced by infection with a virus disabled for VP16, ICP27 and ICP4, western blots to detect levels of ICP4 expression were carried out in the presence and absence of 1764 27-4 virus infection. These western blots are shown in figure 4.9.1A. It can be seen that only very low levels of ICP4 can be detected in the cell line 27/12/27:4 even with viral infection or in the presence of HMBA. This correlates well with the growth curves in figure 4.7.2 which show that this cell line is unable to support effective growth of viruses deleted for ICP4. In contrast, both the 27/12/4:4 and the 27/12/M:4 cell lines show high levels of ICP4 expression, particularly after infection or in the presence of 3mM HMBA. In the case of the 27/12/M:4 cell line, ICP4 levels are even further elevated in the presence of 1 $\mu$ M dexamethasone. It is interesting to note that the 27/12/4:4 cell line has a much higher background expression of ICP4 than cell line 27/12/M:4 (compare lane 10 to lane 6 in figure 4.9.1A). This may be due to the hyper-responsiveness of the ICP4 promoter to the low levels of EHV1 gene 12 continually being expressed by the cell line. This high basal level of toxic ICP4 might be expected to make the 27/12/4:4 cell line unstable and would potentially result in the loss of ICP4 after a limited number of passages. For this reason, work with the 27/12/4:4 cell line was discontinued and 27/12/M:4 was selected for use in routine propagation of multiply disabled viruses.

The MMTV promoter is inducible by the steroid hormone dexamethasone (Lee *et al.*, 1981). Indeed, the cell line using the MMTV promoter to drive ICP4 expression was designed to be used in the presence of dexamethasone following virus infection. Further analysis on the 27/12/M:4 cell line was carried out in order to determine the effect of inclusion of 1 $\mu$ M dexamethasone in the media at the time of infection. Figure 4.9.1A shows that addition of 1 $\mu$ M dexamethasone does indeed induce the MMTV promoter in the context of the cell line 27/12/M:4 (as demonstrated by increased expression of ICP4 in those samples treated with the hormone, for example lane 1 shows greater ICP4 expression than lane 5). It was therefore somewhat surprising to

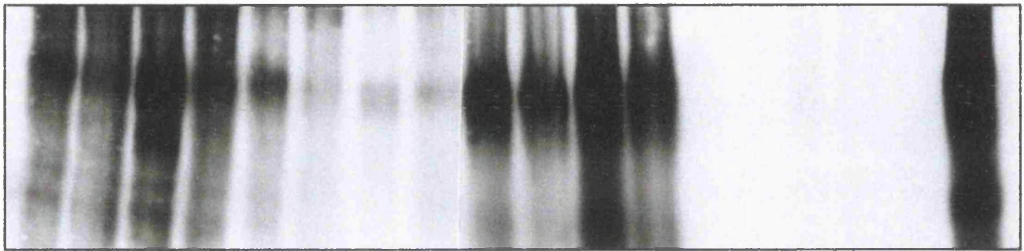
find that inclusion of dexamethasone in the media at the time of infection did not increase the yields of an ICP4 deleted virus obtainable from this cell line. There is some recent evidence to suggest that dexamethasone inhibits viral replication in a rat model of HSV1 encephalitis, potentially providing an explanation for these results (Thompson *et al.*, 2000). The effects of dexamethasone induction are demonstrated by the growth curves in figure 4.9.1B. It can be seen from these growth curves that the greatest yield of VP16, ICP27 and ICP4 was achieved in the presence of 3mM HMBA but without dexamethasone. It is well known that ICP4 turns off its own synthesis (DeLuca and Schaffer, 1988; Roberts *et al.*, 1988; Michael and Roizman, 1993), suggesting that tight regulation of its expression is required for efficient viral growth. It is therefore possible that whilst dexamethasone can induce the MMTV promoter and cause elevated expression of ICP4, this is not at an optimal time during the lytic cycle. Infection, however, is also able to induce the MMTV promoter and here the induction is both temporally and stoichiometrically controlled by the incoming virus, facilitating regulation of gene expression in a more optimal manner.

**Figure 4.9.1      Levels of ICP4 expressed in cell lines 27/12/M:4, 27/124:4 and 27/12/27:4**

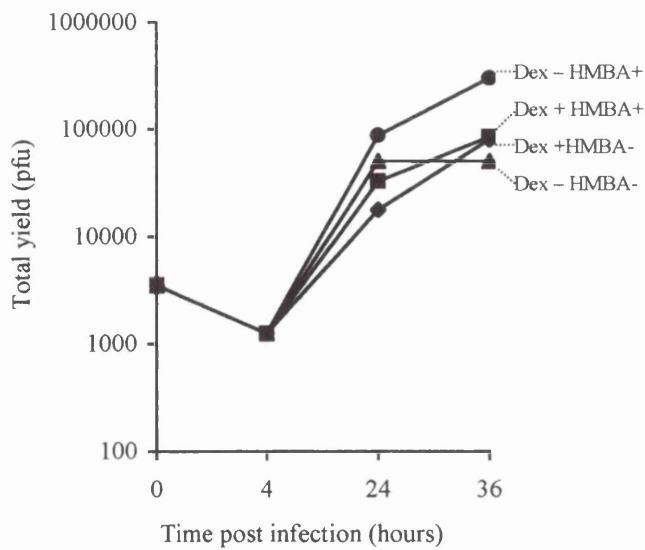
A) Western blot showing induction of ICP4 expression levels from ICP4 containing cells. The cell lines were infected where indicated at an MOI of 5 with a virus deficient in VP16, ICP27 and ICP4. 3mM HMBA or 1 $\mu$ m dexamethasone were included at the time of infection where indicated. Samples were harvested 48 hours post infection. The positive control was a virus deficient in only VP16 and ICP27. After longer exposure, low level expression was also observed from the 27/12/27:4 cells infected with virus and in the presence of HMBA. B) Growth curves to show the effects of dexamethasone on the growth of a virus deficient in VP16, ICP27 and ICP4 on cell line 27/12/M:4. Infections were carried out at an MOI of 0.01 in 24-well plates and yields are shown as total pfu per well (500 $\mu$ l).

**A) ICP4 and MMTV promoters are inducible by virus infection**

Cell line:	27/12/M:4								27/12/4:4				27/12/27:4				BHK
Infection:	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
HMBA:	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+
Dex:	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-



**B) Dexamethasone does not enhance virus growth on cell line 27/12/M:4**

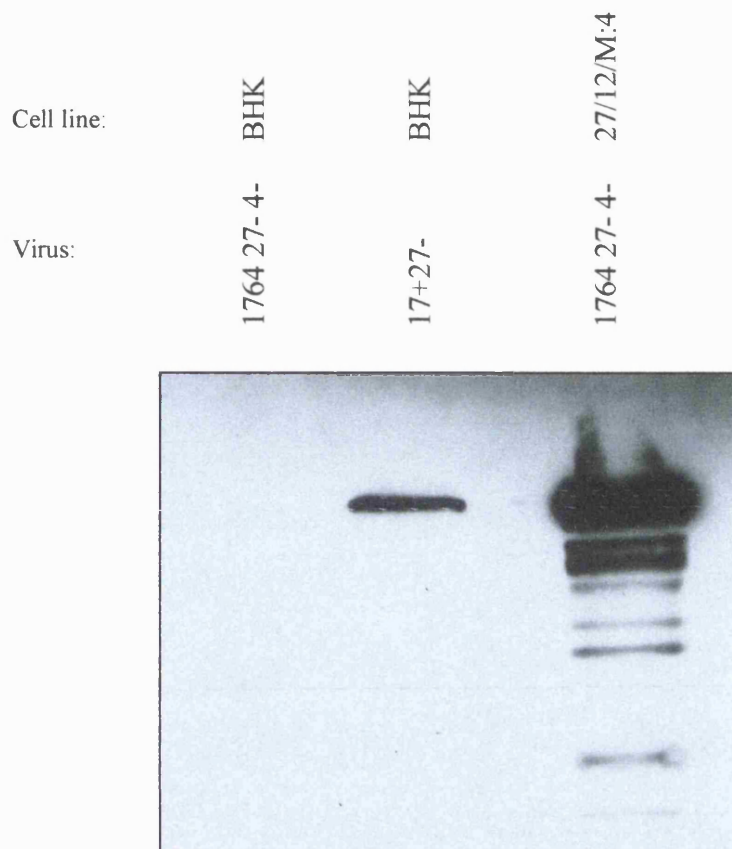


#### **4.10 Viruses deficient in VP16, ICP27 and ICP4 do not express ICP0 on non-complementing cells but are induced to express ICP0 during growth on 27/12/M:4 cells**

In the preceding sections it has become apparent that in the context of the cell lines described, a number of cellularly encoded HSV1 and heterologous promoters are only activated in response to viral infection. It would therefore appear that a factor provided by the incoming virus transactivates these promoters at the time of infection. In transient expression systems, the IE gene ICP0 has been found to be a promiscuous transactivator of a wide variety of promoters and this effect is more pronounced in the presence of ICP4 (Everett, 1984; Gelman and Silverstein, 1985). Activation of gene expression by ICP0 is thought to occur at the level of mRNA synthesis but the exact mechanism by which this occurs is unknown (Samaniego *et al.*, 1997; Jordan and Schaffer, 1997). ICP0 itself does not bind directly to DNA (Everett *et al.*, 1991) so it is assumed that its ability to transactivate genes is mediated through an indirect mechanism. Everett and colleagues have used mutational analysis to identify an N-terminal protein sequence motif which appears to be important in the ability of ICP0 to activate gene expression (Everett *et al.*, 1995). These RING finger motifs are thought to mediate protein-protein interactions (Freemont *et al.*, 1991; Freemont, 1993). This has led to a search for proteins which interact with ICP0 and may facilitate the effects on gene expression. The candidate proteins which have been proposed include proteases involved in ubiquitination and protein degradation (Everett *et al.*, 1998a; Everett *et al.*, 1998b) cyclins and other components of the cell cycle regulatory machinery (Hobbs and DeLuca, 1999). Given these proposed interactions with the ubiquitination pathway, it is possible that ICP0 affects the stability of specific cellular proteins, thereby altering the balance of transcription factors available within the cell and leading to the observed effects on gene activation.

Figure 4.10.1 shows a western blot of ICP0 expression from a VP16, ICP27 and ICP4 deficient virus on BHK cells and on 27/12/M:4 cells. It can be seen from the figure that there is no expression of ICP0 from the disabled virus on BHK cells but on the 27/12/M:4 cells, ICP0 is abundantly expressed. Given the results of this western blot and the foregoing discussion, it seems reasonable to assume that ICP0 is responsible for

the transactivation of the promoters driving the expression of EHV1 gene 12, ICP27 and ICP4 in the cell lines described in this chapter. A proposed model for gene expression from the 27/12/M:4 cells is presented in figure 4.10.2.



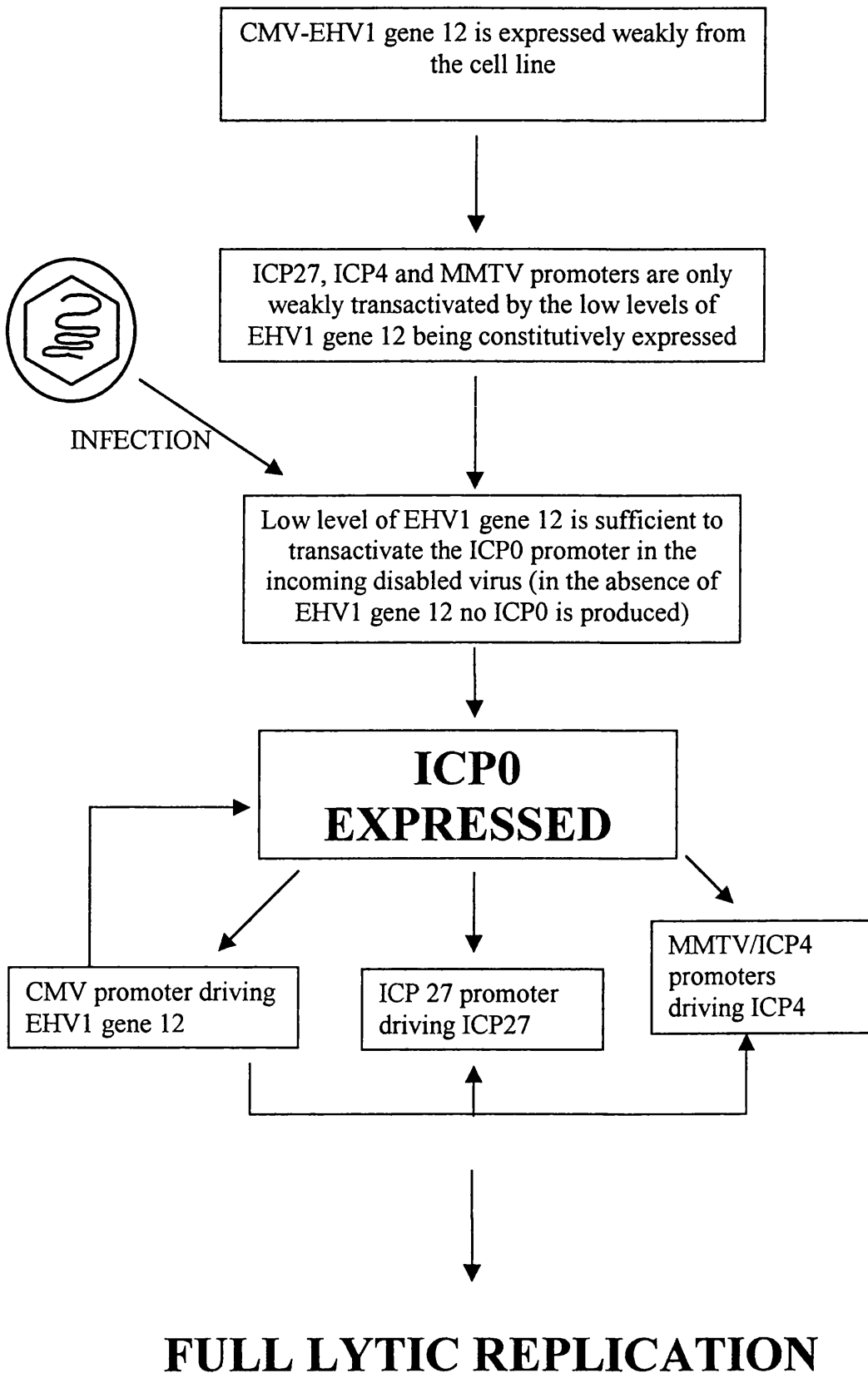
**Figure 4.10.1 Expression of ICP0 from a virus deficient in VP16, ICP27 and ICP4 on BHK cells and on cell line 27/12/M:4**

The western blot shows that there is no expression of ICP0 from the disabled virus on BHK cells but that ICP0 is efficiently produced from this virus when it is grown on cell line 27/12/M:4. The cells were infected at an MOI of 1 and samples were harvested 48 hours post infection.

**Figure 4.10.2 Model of gene expression in 27/12/M:4 cells**

In the absence of viral infection, EHV1 gene 12 is expressed weakly from the CMV promoter and only low levels of ICP4 and ICP27 are produced by the cells. Following infection with a virus deficient in VP16, ICP27 and ICP4, the product of EHV1 gene 12 transactivates the ICP0 promoter. The ensuing expression of ICP0 further activates the EHV1 gene 12 promoter and also activates the MMTV and ICP27 promoters. The full lytic cycle can then proceed.





#### 4.11 Discussion

The arrival of VP16 in the nucleus of a permissive HSV1 infected cell heralds the onset of the lytic gene cascade. The importance of the role of this protein is reflected in the fact that it is conserved in a number of the  $\alpha$ -herpesviruses. Homologues from Varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV1), EHV1 and EHV4 have all been documented (McKee *et al.*, 1990; Carpenter and Misra, 1992; Purewal *et al.*, 1994). All known VP16 homologues share the ability to transactivate IE gene promoters and all are tegument phosphoproteins yet they differ in their structures and functional layouts. The HSV1 VP16 protein is clearly divided into two domains. The N-terminus mediates DNA binding through its interaction with the Oct1 homeodomain, and the acidic carboxy terminus is necessary and sufficient for the transactivation function (Ace *et al.*, 1988; Triezenberg *et al.*, 1988). VP16 homologues from VZV and EHV1 are not so clearly divided in their functional layout. These proteins completely lack the C-terminal domain but are still fully functional transactivators. Mutational analysis and construction of fusion proteins has revealed that in the case of EHV1 at least, no single domain is sufficient for its transactivational function (Elliott, 1994).

Despite this apparent structural diversity, the family of VP16 homologues have retained the ability to perform near identical functions in their respective viruses. The idea of functionally substituting one for another is therefore an extrapolation of this observation. Such substitutions have been carried out in the past but usually in a purely academic context, the less studied (EHV and BHV) or less easily studied (VZV)  $\alpha$ -herpesviruses often benefiting from the volume of work published on HSV1 VP16. The work presented in this chapter demonstrates that the product of EHV1 gene 12 can fully substitute for the transactivation function of HSV1 VP16. Importantly, the data presented in section 4.5 of this chapter demonstrate that the product of EHV1 gene 12 is not packaged into HSV1 virions, meaning that this protein cannot compensate for the essential structural role of HSV1 VP16.

Evolutionarily, the VP16 homologues present an interesting question. How have the proteins diverged so much in sequence and functional layout but yet retained the ability to functionally substitute for one another? The answer might lie in the fact that each of the VP16 homologues are structural proteins as well as transactivators. On the

one hand the proteins hold a somewhat inflexible position at the top of a gene cascade yet on the other hand they are continually challenged to evolve to avoid the host's immune response. None of the VP16 homologues can themselves directly bind DNA with high efficiency and are therefore reliant on complexing with cellular factors in order to carry out their function. HSV1 VP16 and homologues from EHV1 and BHV1 have been shown to form a complex with the cellular proteins Oct1 and HCF (host cell factor) on TAATGARAT-like consensus sites in target gene promoters. It is therefore possible that the role of the VP16 homologues as structural proteins has forced their considerable sequence divergence but the ability to interact with the same host cell factors is an element which all the homologues have conserved by necessity and a possible reason for their cross-species promiscuity.

The work described in this chapter details the construction of a number of cell lines designed to support the growth of an HSV1 virus with deficiencies in the expression of multiple IE genes. Two factors were found to be important in the construction of such cell lines. Firstly, the necessity for a level of complementation of VP16 deficiencies over and above that provided by HMBA, and secondly the choice of promoter driving the cellular expression of HSV1 genes. An optimal cell line was constructed using CMV driven EHV1 gene 12, ICP27 driven ICP27 and MMTV driven ICP4. The promoters controlling the expression of all these genes were found to be induced in response to virus infection and this was presumed to be controlled by transactivation of the incoming ICP0 gene by the product of EHV1 gene 12. The construction of this cell line was only possible due to the fact that the virus described in chapter 3 had been previously constructed. Being deficient in VP16, ICP27 and ICP4 this virus provided a means of screening colonies and selecting an optimal cell line. As detailed in chapter 3, this virus is unstable due to homologous recombination between repeated elements and cannot therefore be propagated in any quantity. However, generation of this virus broke the vicious circle of on the one hand having no cell line to adequately grow multiply disabled virus and on the other having no multiply disabled virus with which to screen for such a cell line. Virus 1764 27- 4- pR20.5 has therefore served its purpose and was not used again in this thesis.

Using the 27/12/M:4 cell line, it was anticipated that it would now be possible to construct a multiply disabled virus which could be used as a stable backbone for future insertions of transgene containing cassettes. The construction of this stable backbone vector is described in the next chapter.

## **CHAPTER 5**

# **CONSTRUCTION OF A STABLE VECTOR BACKBONE**

## 5.1 Introduction

Studies of defective interfering (DI) particles from HSV1 have demonstrated only too well the significant growth advantage that can be conferred by having a smaller genome (Bronson *et al.*, 1973). Compound this with an inefficient complementing cell line forcing the use of high MOIs together with multiple copies of the LAT P2 region, and the resulting viruses have levels of genome instability which are unacceptably high. Such was the problem with the viruses described in chapter 3.

Chapter 4 resolved the problem of the complementing cell line and this chapter aimed to overcome the instability which results from genomes harbouring excess copies of the LAT P2 region. There are two possible ways in which this problem could be solved. The first is to always insert transgenes directly into the LAT regions themselves, thereby using LAT P2 to confer long term expression from its endogenous position within the viral genome. The second is to delete both copies of the endogenous LAT P2 region from the viral backbone and insert a LAT P2 containing cassette at any ectopic site. In the interests of versatility and as the project required the high level expression of pairs of genes, it was the latter approach which was pursued in this chapter.

The strategy employed in order to generate a stable, ICP4 deleted viral backbone is summarised schematically in figure 5.1. Firstly, a *lacZ* containing cassette was inserted into the LAT region of the stable virus 1764 27-. Next a plasmid containing DNA which flanked the LAT P2 region was recombined into this virus, “knocking out” the *lacZ* cassette and the endogenous LAT P2. This created a white virus with no copies of LAT P2 which was then used as a starting point for the deletion of ICP4. ICP4 was deleted by the insertion of an MSV-GFP cassette and then this cassette was “knocked out” as before. The end result was therefore a 1764 27- 4- white virus with no copies of LAT P2. The plasmids and viruses which were constructed in order to facilitate the production of this stable virus are described in this chapter.

**Figure 5.1.1 Schematic flow diagram to illustrate the strategy employed to generate a stable, fully disabled viral genome**

A flow chart is shown to schematically illustrate the viruses which were constructed in order to remove both copies of LAT P2 and ICP4 from the viral genome.

1. A CMV-*lacZ* containing cassette was inserted into the LAT region of the stable virus 1764 27-.
2. A plasmid containing DNA which flanked the LAT P2 region was recombined into this virus, “knocking out” the *lacZ* cassette and the endogenous LAT P2.
3. A MSV-GFP cassette was inserted into the ICP4 locus of this stable white virus.
4. The MSV-GFP cassette was “knocked out” to generate a white version of the stable fully disabled virus.



1764 27- viral genome with both endogenous LAT P2 regions intact  
Colour = white



1764 27- viral genome with a CMV *lacZ* cassette inserted downstream of both endogenous LAT P2 regions  
Colour = blue



1764 27- viral genome with both endogenous LAT P2 regions deleted  
Colour = white



1764 27- 4- viral genome with both endogenous LAT P2 regions deleted and a MSV-GFP cassette inserted into both copies of ICP4.  
Colour = green



1764 27- 4- viral genome with both endogenous LAT P2 regions and both copies of ICP4 deleted  
Colour = white



## 5.2 Insertion of a CMV-*lacZ* cassette into the LAT region

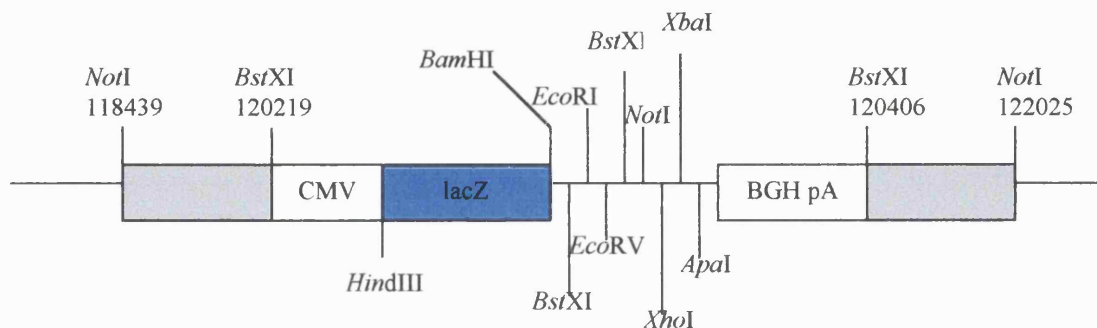
The expression cassette pR19*lacZ* contains a CMV promoter driving the expression of the *lacZ* gene in LAT flanking regions. The plasmid had been previously constructed in our laboratory as described below. The *lacZ* gene from pCH110 (Pharmacia) was excised using *Hind*III and *Bam*HI and cloned between the *Bam*HI and *Hind*III sites in pcDNA3 (Invitrogen). This placed the *lacZ* gene under the control of the CMV promoter and BGH polyadenylation signal. The LAT flanking regions were derived from the *Not*I region of the HSV1 genome (nt 118439-122025). This genomic fragment was cloned into the unique *Not*I site of the cloning vector pGem5 (Promega) to create plasmid pNot3.5. The *Bst*XI site in the pGem5 backbone was deleted by digestion with *Sac*I and *Nsi*I (sites flanking *Bst*XI), treatment with T4 DNA polymerase and religation. The CMV-*lacZ*pA cassette was then excised from pcDNA3 using *Nru*I and *Bbs*I and inserted into pNot3.5 between the *Bst*XI sites at HSV1 nucleotides 120219 and 120406. A map of plasmid pR19*lacZ* is shown in figure 5.2.1A.

pR19*lacZ* was linearised using a unique *Xmn*I site in the plasmid backbone and cotransfected with infectious 1764 27- viral DNA. Recombinants were identified by their ability to express *lacZ* and plaque purified. A map of the virus 1764 27- pR19*lacZ* is shown in figure 5.2.1B. It can be seen from the figure that the recombination places the CMV promoter immediately 3' of the endogenous LAT P2 region.

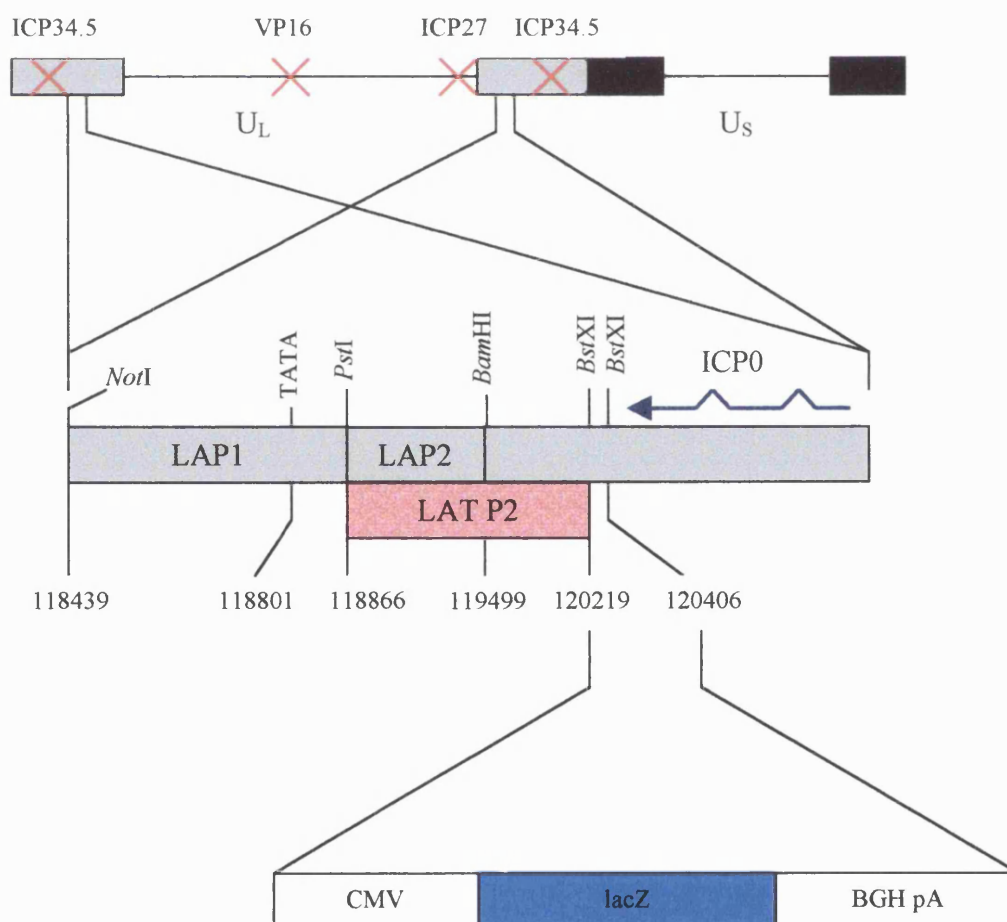
**Figure 5.2.1 Maps of plasmid pR19*lacZ* and virus 1764 27- pR19*lacZ***

A) Map of the pR19*lacZ*/Not3.5 plasmid in pGEM5. The CMV-*lacZ* cassette was cloned into LAT flanking regions as described in section 5.2. Regions homologous to the long repeats of the HSV1 genome are shown in grey and nucleotide numbers are indicated for orientation. B) Map of the virus 1764 27- pR19*lacZ*. Following homologous recombination with infectious viral DNA, the CMV *lacZ* cassette is inserted immediately 3' of the LAT P2 region.

**A) Map of the pR19lacZ/Not3.5 plasmid in pGEM5**



**B) Map of the virus 1764 27- pR19lacZ**



### 5.3 Removal of the CMV-*lacZ* cassette and the LAT P2 region

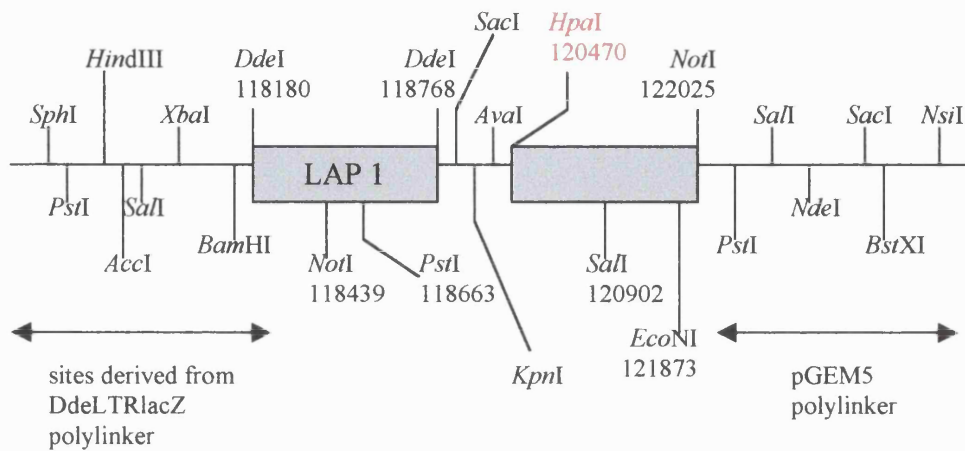
In order to remove the LAT P2 region and the CMV *lacZ* cassette from virus 1764 27- pR19*lacZ*, plasmid pΔP2 was constructed. This plasmid contains regions of DNA which flank the LAT P2 region in the viral genome. Plasmid pDdeRev was previously constructed in the laboratory and contains a *DdeI* restriction fragment from the HSV1 genome (nt 118180-118768) encoding LAP1 up to but not including the TATA box. The *DdeI* fragment from this plasmid was excised as a *EcoRI/SphI* fragment and cloned into pNot3.5 between the *HpaI* site at HSV1 nucleotide 120470 and the *SphI* site in the pGEM5 polylinker. The *HpaI-NotI* fragment (nt 120470-122025) from the pNot3.5 plasmid then became the 3' flanking region of pΔP2 and the *DdeI* fragment (118180-118768) excised from pDdeLTRL*lacZ* became the 5' flanking region. A map of plasmid pΔP2 is shown in figure 5.3.1A.

Viral DNA was prepared from 1764 27- pR19*lacZ* as described in section 2.4.7. Plasmid pΔP2 was linearised using a unique *XmnI* site in the plasmid backbone and cotransfected with this infectious viral DNA. After staining with X-Gal, recombinants were identified as white plaques and purified away from a background of blue plaques. The resulting virus was named 1764 27- P2-. The structure of this virus is shown in figure 5.3.1B.

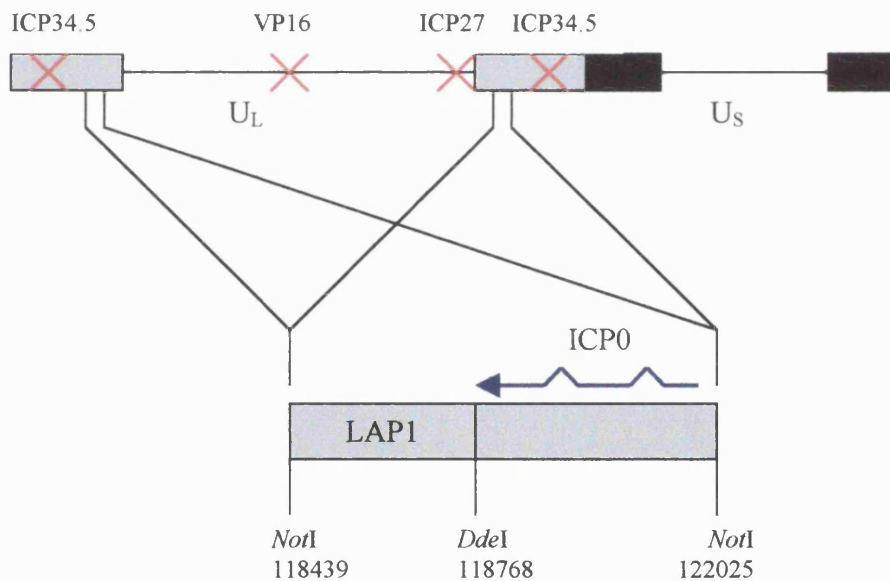
During negative selection (i.e. when white plaques are being selected from a coloured background), there is an increased chance that the selected recombinant may not be of the desired structure. In the above example, a white plaque could arise as a result of a spontaneous mutation in the reporter gene rather than deletion of the entire cassette plus LAT P2. Therefore when reporter genes are not used, it is particularly important to confirm the insertion or deletion of the desired region of DNA by Southern blotting. In order to confirm the structure of this virus, a Southern blot was carried out. Viral DNA from viruses 1764 27- and 1764 27- P2- was prepared as described in section 2.4.6 and cut with *NotI*. The DNA was transferred to Hybond N and probed with either the LAT P2 region (*PstI-BstXI*, nt 118866-120219) or the entire *NotI* fragment (nt 118439-122025) from pNot3.5. The Southern blot is shown in figure 5.3.2. It can be seen that in the case of virus 1764 27-, the 3.5Kb *NotI* fragment can be detected.

However, in the case of 1764 27- P2-, the 3.5kb *NotI* fragment was reduced in size by approximately 1.5kb, corresponding to the size of the deleted LAT P2 region. Similarly, when the blots were probed with the LAT P2 region, a 3.5kb fragment hybridised in the case of 1764 27- but not in the case of 1764 27- P2-. The structure of 1764 27- P2- was therefore confirmed to be correct.

**A) Map of pΔP2**

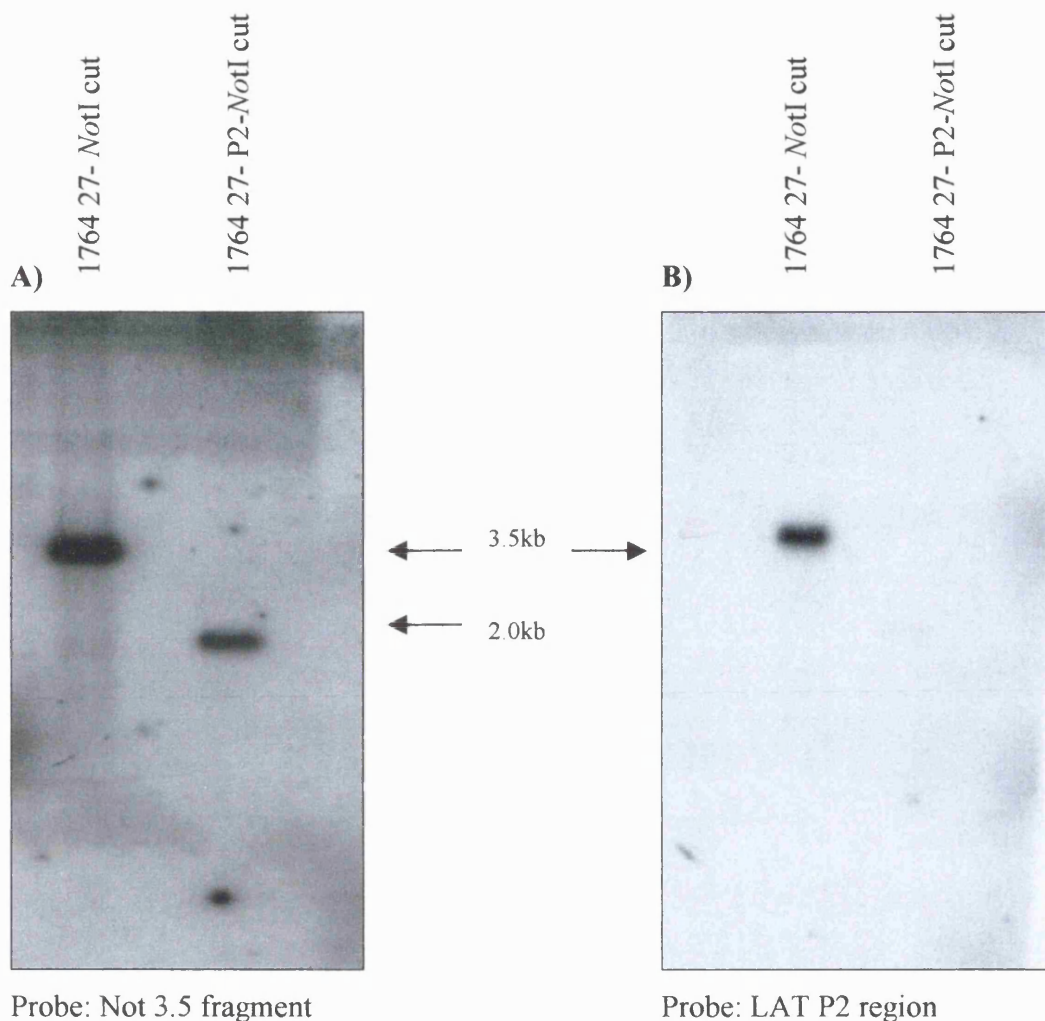


**B) Map of 1764 27- P2-**



**Figure 5.3.1 Structures of the plasmid pΔP2 and the virus 1764 27- P2-**

A) Map of pΔP2. This plasmid was designed to delete the endogenous LAT P2 region. The *HpaI* site shown in red is marked on the map for orientation purposes only, it has been destroyed in the cloning. B) Map of the virus 1764 27- P2-. It can be seen the LAT P2 region (between nucleotides 118768 and 120470 in the case of the long internal repeat shown) has been deleted from both the long repeat regions of the viral genome.



**Figure 5.3.2 Southern blot to confirm the deletion of the LAT P2 region**

Viral DNA prepared from 1764 27- and 1764 27- P2- was cut with *NotI* and probed with the *NotI* fragment excised from pNot3.5 (HSV1 nt 118439-122025). In the case of 1764 27-, this probe hybridises with the identical 3.5kb fragment whereas in the case of 1764 27-P2-, it hybridises with a fragment which is approximately 1.5kb smaller. This size reduction corresponds to the size of the deleted LAT P2 region. B) Viral DNA prepared from 1764 27- and 1764 27- P2- was cut with *NotI* and probed with the LAT P2 region. It can be seen from the blot that a 3.5kb corresponding to the *NotI* fragment containing LAT P2 hybridises in the case of 1764 27- but not in the case of 1764 27-P2-.

#### 5.4 Recombination using pΔP2 would prevent expression of a transcript antisense to ICP0

The *DdeI* site which marks the 3' end of the 5' flanking region of pΔP2 is at HSV1 nucleotide 118768. It can be seen from figure 5.2.1 that the TATA box of LAP1 is at nucleotide 118801. Use of pΔP2 to remove the endogenous LAT P2 region will therefore also result in the loss of the TATA box of LAP1. This would be expected to prevent the production of any of the LAT transcripts and thus result in no RNA antisense to ICP0. It was anticipated that this might be undesirable in situations when ICP0 was considered to be contributing to vector cytotoxicity. A second plasmid, pΔP2(+P1) was therefore constructed. pΔP2(+P1) was made by deleting the pGEM5 polylinker from pΔP2 by cutting with *EcoNI* and *NsiI*, treating with T4 DNA polymerase and religating. A *NotI*/*StyI* fragment (nt 118439-118876) was cut out of pNot3.5 and cloned between the *SacI* (polylinker derived) and *NotI* (nt 118439) sites of pΔP2 (now minus pGEM5 polylinker). This essentially extends the 5' flanking region of pΔP2 by 100 nucleotides so that it now contains the TATA box of LAP1. The structure of pΔP2(+P1) is shown in figure 5.4.1A.

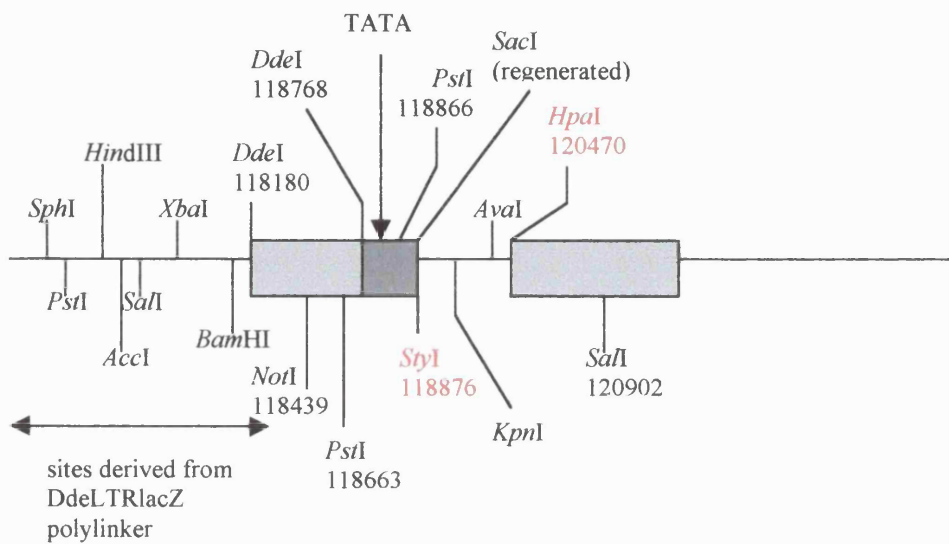
In order to facilitate exchange between pΔP2 and pΔP2(+P1), a final plasmid in this set was constructed. This was MSV*lacZ*/pΔP2. The MSV promoter was used so that the exchange could be carried out after the insertion of CMV containing cassettes elsewhere in the viral genome without the risk of aberrant recombination events. The structure of plasmid pMSV*lacZ*/ICP4 is shown in figure 3.11.1. The MSV*lacZ* cassette was excised from the pBS MSV-*lacZ* (described in section 3.11) using *NotI* and *XhoI*, treated with T4 DNA polymerase and inserted into the *KpnI* site of pΔP2. A map of pMSV*lacZ*/pΔP2 is shown in figure 5.4.1B. It later became apparent that these concerns about lack of antisense to ICP0 were largely irrelevant in the context of a virus with ICP4 deleted (see chapter 6) so plasmids pΔP2(+P1) and MSV*lacZ*/pΔP2 were not used in this thesis.



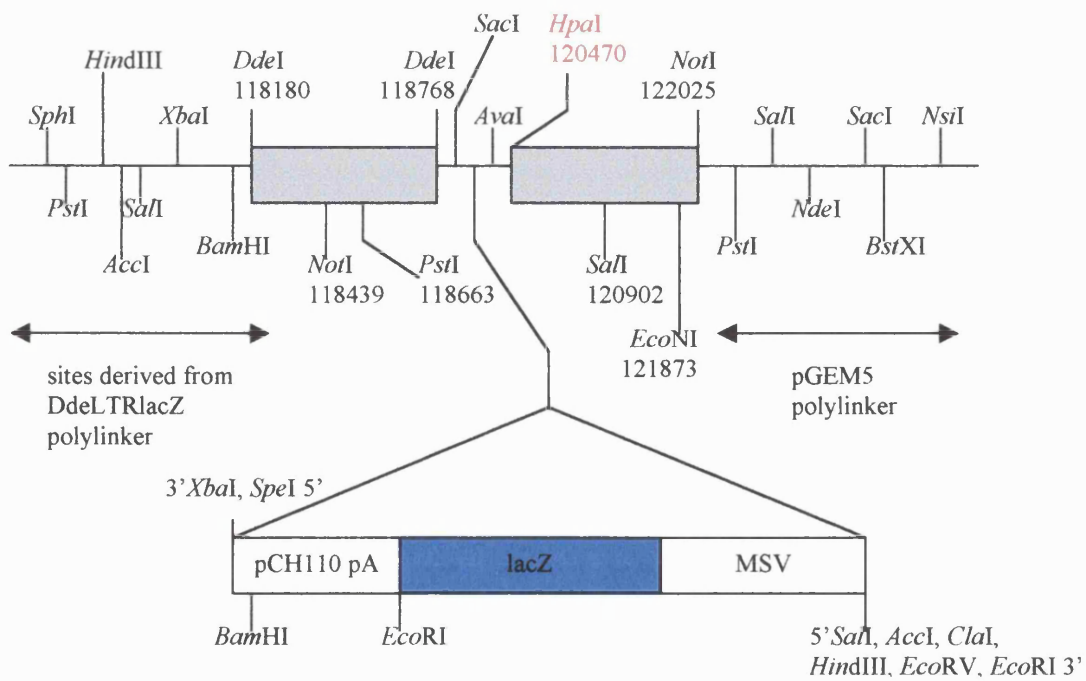
**Figure 5.4.1 Maps of plasmids pΔP2(+P1) and pMSV-*lacZ*/pΔP2**

A) pΔP2(+P1) was constructed in order to restore production of a RNA transcript antisense to ICP0. This plasmid is the same as pΔP2 except that the 5' flanking region is extended by 100 base pairs (indicated by the darker grey area). The *Hpa*I and *Sty*I sites shown in red are indicated for orientation purposes only, they have been destroyed in the cloning. B) pMSV-*lacZ*/pΔP2 was constructed by insertion of the MSV-*lacZ* cassette into the unique *Kpn*I site of pΔP2. This plasmid was designed in order to facilitate exchange between pΔP2 and pΔP2(+P1).

### A) pΔP2(+P1)



### B) MSV-lacZ/pΔP2



## 5.5 Deletion of ICP4 from 1764 27- P2-

In order to facilitate the removal of ICP4 from the virus 1764 27- P2-, plasmid pMSV-GFP/4 was constructed. This plasmid consists of the MSV promoter driving expression of the reporter gene GFP in the ICP4 flanking regions. GFP was chosen as the marker gene for this stage as it enables the picking of plaques directly through carboxymethylcellulose (CMC, see section 2.4.3), thus reducing the potential of mixing the different viral populations as might occur during washing and staining with X-Gal. It was anticipated that this might be important in this case as the recombinant virus (ICP4 deleted) could still have a growth disadvantage over the backbone virus (ICP4 not deleted).

The plasmid pMSV-GFP/4 was constructed as follows. The GFP cassette from pEGFPN1 (Clontech) had been previously been inserted into the unique *NotI* site in the pcDNA3 polylinker. The CMV-GFP-pA cassette from pcDNA3 had then been excised as a *NruI*-*BbsI* fragment and inserted into the unique *SmaI* site in pSP72 generating pSP72CMV-GFP-pA. Both of these plasmids previously existed in the laboratory. The GFP-pA cassette was then excised from pSP72CMV-GFP-pA as a *PstI* fragment and inserted in place of *lacZ* between the *BamHI* sites in plasmid pBSMSV-*lacZ* (see section 3.11). This created pBSMSV-GFP, the structure of which is shown in figure 5.5.1A. The MSV-GFP cassette was excised from pBSMSV-GFP as a *ClaI*/*XhoI* restriction fragment and inserted between the *ClaI* and *EcoRI* restriction sites in pMSV-*lacZ*/4 (construction of which was previously described in section 3.11). This generated plasmid pMSV-GFP/4. A map of this plasmid is shown in figure 5.5.1B.

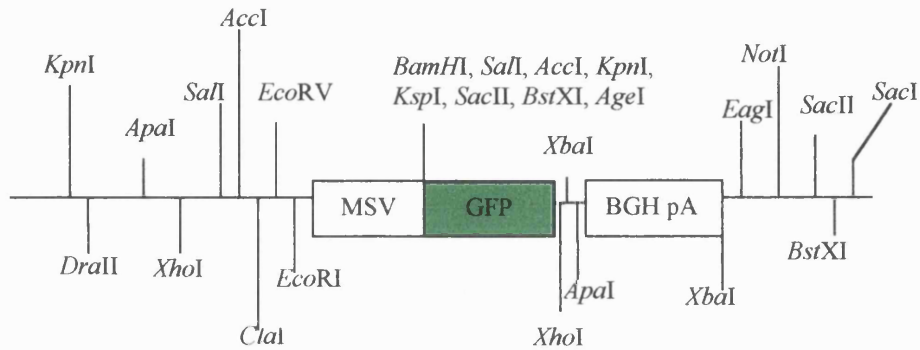
pMSV-GFP/4 was linearised using a unique *ScaI* site in the plasmid backbone and cotransfected with infectious viral DNA prepared from 1764 27- P2-. The transfection was carried out on cell line 27/12/M:4, the construction of which was described in chapter 4. Recombinants were identified by their ability to express GFP and the virus was plaque purified. Interestingly, two recombinant populations arose from this transfection, represented by bright green or faint green plaques. This was assumed to be a consequence of there being two copies of ICP4 in the viral genome, the faint and bright plaques representing insertion of GFP at one or both ICP4 loci

respectively. Based on this assumption, the brighter plaques were preferentially selected during purification. Unlike the previous attempted purification of an ICP4 deleted virus (section 3.11), virus 1764 27- P2- 4- MSV-GFP/4 purified easily. This was considered to be attributable to the new complementing cell line 27/12/M:4. The structure of the virus 1764 27- P2- 4- MSV-GFP/4 is shown in figure 5.5.1C.

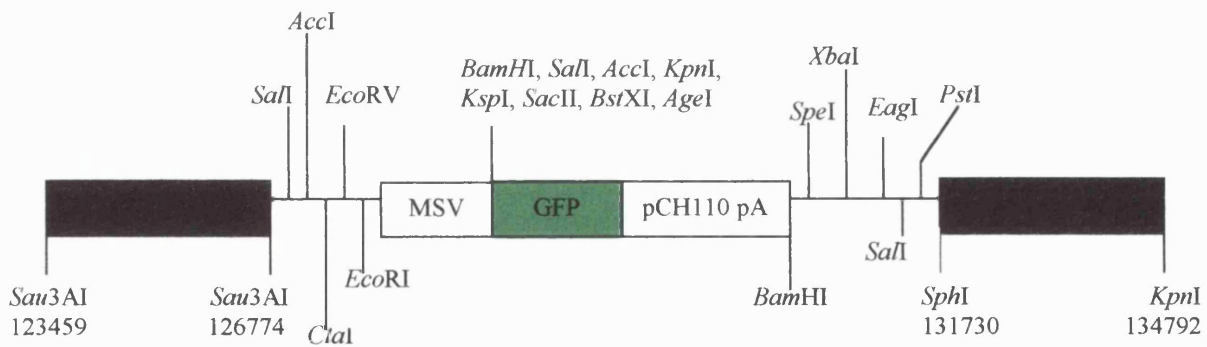
**Figure 5.5.1 Maps of pBSMSV-GFP, pMSV-GFP/4 and virus 1764 27- P2- 4- MSV-GFP/4**

A) A map of plasmid pBSMSV-GFP is shown. The details of the cloning can be found in section 5.5. B) The shuttle plasmid pMSV-GFP/4. The ICP4 flanking regions are shown in black. This plasmid was generated as detailed in section 5.5 and used to remove both copies of ICP4 from the virus 1764 27- P2-. C) Map of the virus 1764 27- P2- 4- MSV-GFP/4. It can be seen that following homologous recombination, the MSV-GFP cassette is inserted into the ICP4 loci in the viral genome.

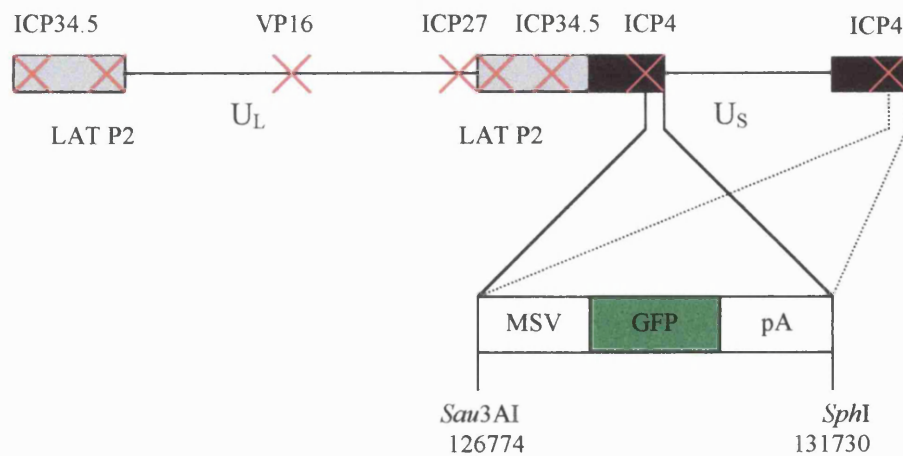
### A) pBSMSV-GFP



### B) pMSV-GFP/4



### C) Map of virus 1764 27- P2- 4- MSV-GFP/4

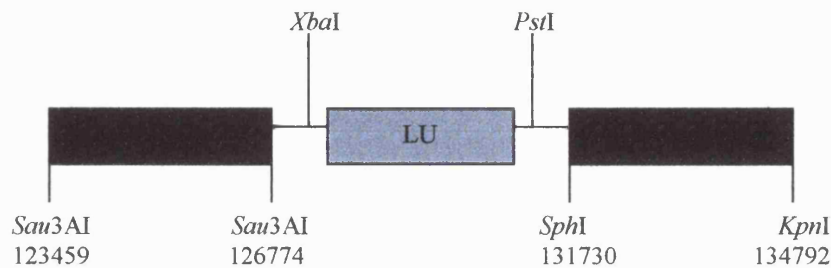


## 5.6 Deletion of GFP from the virus 1764 27- P2- 4- MSV-GFP/4

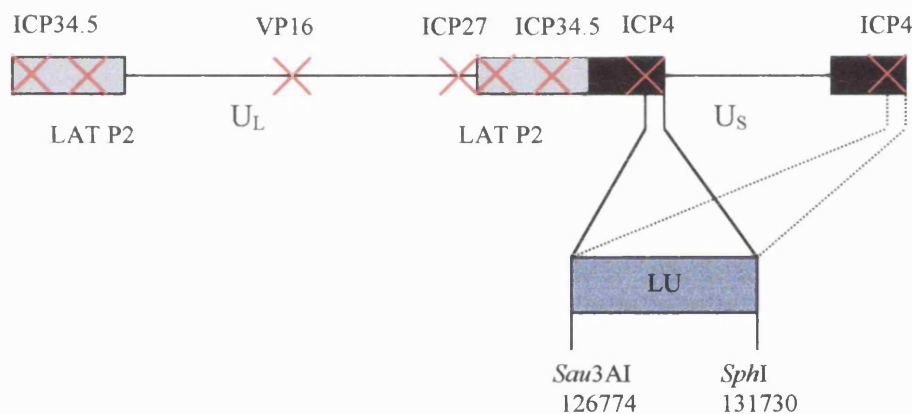
In order to generate a stable ICP4 deleted virus which could act as a starting point for insertion of the bicistronic expression cassettes described in chapter 3, the MSV-GFP cassette must be deleted from virus 1764 27- P2- 4- MSV-GFP/4. This was achieved using plasmid pLU/4. This plasmid consists of 100 base pairs of the reporter gene luciferase cloned into the ICP4 flanking regions. The rationale behind the design of this plasmid was to construct an insert which could be used as a marker but without the use of a promoter or pA sequence which may lead to recombination problems later. The 100 base pair fragment could have been from any known sequence of DNA, its only purpose being to be identifiable by Southern blot. This system can be used at any later stage in virus construction to confirm that the ICP4 deletion has been maintained. pLU/4 was constructed by inserting a *Bsa*AI/*Xba*I fragment from pGL2 Basic (Promega) between the *Sal*I and *Xba*I sites of the ICP4 flanking regions plasmid, the construction of which is described in section 3.9. A map of pLU/4 is shown in figure 5.6.1A.

Plasmid pLU/4 was linearised using a unique *Xmn*I site in the plasmid backbone and cotransfected with infectious 1764 27- P2- 4- MSV-GFP/4 viral DNA. Recombinant plaques were identified by their inability to express GFP and these white plaques were purified away from a background of green plaques. A map of the resulting virus, 1764 27- P2- 4- LU/4 is shown in figure 5.6.1B. As this virus did not express a reporter gene, the structure was confirmed to be correct by Southern blotting. Viral DNA was prepared from 1764 27- P2- 4- LU/4 and 1764 27- P2- 4- MSV-GFP/4 and cut with *Xba*I/*Sph*I. The 100 base pair *Xba*I/*Sph*I fragment from pLU/4 was used as a probe and a positive control. This Southern blot is shown in figure 5.6.2.

### A) Map of plasmid pLU/4



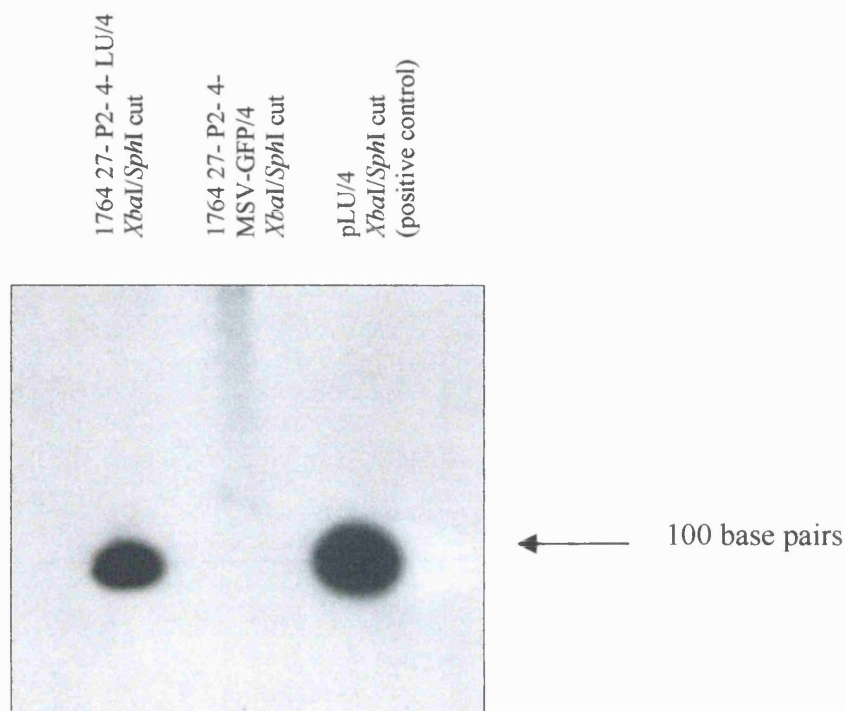
### B) Map of virus 1764 27- P2- 4- LU/4



**Figure 5.6.1 Maps of plasmid pLU/4 and virus 1764 27- P2- 4- LU/4**

A) Map of plasmid pLU/4. This plasmid was constructed by inserting a 100 base pair fragment of luciferase into the ICP4 flanking regions. The cloning is described in section 5.6. The fact that the oligonucleotide came from luciferase is irrelevant, it is merely acting as a marker for successful removal of the MSV-GFP cassette and continuing lack of ICP4. B) Map of virus 1764 27- P2- 4- LU/4. The insertion of the 100 base pair luciferase fragment into the ICP4 locus is indicated.





**Figure 5.6.2 Southern blot of virus 1764 27- P2- 4- LU/4**

Viral DNA was prepared from viruses 1764 27- P2- 4- LU/4 and 1764 27- P2- 4- MSV- GFP/4 and cut with *XbaI* and *SphI*. The cut DNA was transferred to Hybond N as described in section 2.2.15 and probed with the *XbaI/SphI* fragment from pLU/4. The positive control was pLU/4 cut with *XbaI* and *SphI*. It can be seen that the probe hybridises to an appropriately sized fragment in the virus 1764 27- P2- 4- LU/4, confirming the presence of the luciferase insert.

## 5.7 Discussion

The ability to establish a life-long latent infection after a single application is the promise that HSV1 holds as a gene delivery vector. The stable viruses constructed in this chapter have both copies of the endogenous LAT P2 region deleted. It is therefore important to consider the possibility that introducing such deletions into the endogenous LAT regions will adversely affect the ability of the virus to establish or maintain a latent infection.

Several reports have analysed LAT deletion mutants and concluded that these variants are deficient in reactivation but not in the establishment of latency (Leib *et al.*, 1989; Hill *et al.*, 1990) and that this reactivation function is contained within the first 1.5kb of the 8kb transcript (Perng *et al.*, 1996). This not only suggests that deletion of both copies of the LAT P2 region will not affect the ability of the virus to establish a latent infection but that such deletions might reduce the likelihood of reactivation, adding an additional level of safety. Indeed, the authors of the first report to demonstrate expression of a transgene through latency stated that they deleted both copies of the LAT region for precisely this reason (Dobson *et al.*, 1990).

However, the perception that LAT deficient viruses establish latency at levels comparable to wild type viruses was challenged by Thompson and Sawtell in 1997 (Thompson and Sawtell, 1997). These authors observed that LAT deficient viruses were only able to establish a latent infection in approximately 10% of the trigeminal ganglia neurons of infected mice whereas a wild type virus could establish a latent infection in 30% of neurons. The LAT deficient virus was then artificially induced to establish a latent infection as efficiently as the wild type virus (by applying stress during the acute stage of the infection) and the two were compared for their ability to reactivate. The authors observed no differences in reactivation and concluded that any perceived reduction in reactivation from latency which had previously been reported was actually an indirect effect of reduced establishment. The differences between these studies are primarily the latency model used and the methods employed to detect the latent genomes. The latter difference is likely to be the most important since analysis of the assays used reveals a possible difference in sensitivity. The reports which conclude that LATs are important in the reactivation from but not the establishment of latency use

quantitative dot blots whereas Thompson and Sawtell use a PCR method to detect latent genomes in single neurons. It is therefore possible that the deletion of the endogenous LAT P2 regions in the viruses described in this chapter could impair the ability of the viruses to establish latency. If this is the case, it is encouraging that the phenomenon is not noted by most authors, as this indicates that any effect is likely to be subtle. Additionally, the studies described use replication competent viruses whereas the viruses described in this chapter do not replicate so the process of ‘establishment’ of latency (or enabling the viral genome to persist in the long term in infected neurons) may be different.

This chapter describes the construction of a series of viruses, the end point of which is a stable virus deficient in VP16, ICP34.5/ORF P, ICP27 and ICP4 and has both copies of the LATP2 region and TATA box of LAP1 deleted. The virus expresses no reporter genes and is called 1764 27- P2- 4- LU/4. This stable virus can now be used as a starting point for the insertion of the bicistronic expression cassettes containing *lacZ* and GFP and subsequently the genes of interest. The construction and characterisation of viruses containing reporter gene cassettes is described in the next chapter.

## **CHAPTER 6**

# **CONSTRUCTION AND CHARACTERISATION OF STABLE, ICP4 DELETED VIRUSES CONTAINING REPORTER GENE CASSETTES**

## 6.1 Introduction

This chapter describes the insertion of various expression cassettes into the stable and versatile vector backbone described in the previous chapter. These insertions can be broadly divided into two classes, those which place reporter gene cassettes containing LAT elements into non-LAT (ectopic) sites in the genome and those which place reporter gene cassettes under the control of LAT elements in the endogenous LAT regions. This chapter includes examples of viruses constructed by both of these approaches.

The large size of the HSV1 genome has often been suggested as one of the reasons why this virus might be suitable as a gene delivery vector, the capacity to express multiple exogenous genes being a desirable characteristic in a number of applications. However, this capacity has so far only been exploited at the expense of long term transgene expression (Krisky *et al.*, 1998a). No HSV1 vectors allowing the long term expression of multiple exogenous genes have been reported. The bicistronic expression cassettes used in this thesis potentially offer a novel means of taking advantage of the large packaging capabilities of HSV1 without compromising the possibility for long term expression of the transgenes.

The first set of viruses described in this chapter are based on the bicistronic expression cassettes pR20.1 to pR20.9 which were described in chapter 3. Parallel work in our laboratory in which these cassettes were used in an attenuated but replication competent (1764) virus in the peripheral nervous system had suggested that pR20.5 and pR20.9 were promising for *in vitro* and *in vivo* work respectively (for example see Palmer *et al.*, 2000). This conclusion was based on the observation that the expression afforded by the LAP1 and MMLV LTR promoters in pR20.9 increased in intensity several weeks after injection, presumably corresponding to the onset of latency. However, during replication in culture and at short time points post injection *in vivo*, the expression levels were very weak, especially from the LAP1 promoter. This was assumed to be attributable to a LAT P2 dependent repression of the MMLV and LAP1 promoters by ICP4 and/or ICP0 expressed during the lytic cycle. However, following the onset of latency, there is no expression of the IE genes and these repressive effects on the MMLV and LAP1 promoters are relieved. In contrast, the CMV and RSV

promoters in pR20.5 afforded very high levels of expression of both transgenes in culture and at short time points *in vivo*. Whilst expression from pR20.5 was sustainable in the long term, this was at reduced levels from those initially achieved. It therefore appears possible that LAT P2 is necessary, but not entirely sufficient for optimal long term expression and that certain regions of LAP1 might also be required.

Considering this PNS data, it was decided to utilise only these two bicistronic expression cassettes (pR20.5 and pR20.9) for constructing multiply disabled viruses for the delivery of marker or AD-related genes to CNS neurons. It was anticipated that the absence of ICP4 and minimal expression of ICP0 (see sections 5.10 and 6.7) from the virus 1764 27- P2- 4- might mean that the LAP1 and MMLV promoters would not be repressed and that pR20.9 could afford higher level expression in non-complementing cells *in vitro*. Viruses were constructed containing the pR20.5 cassette in order to further characterise the potential of an isolated LAT P2 element to confer long term expression capabilities onto heterologous promoters. Additionally, since one of the aims of this project was to analyse potential interactions between WT and mutant APP and PS1 in culture, high level expression of both genes would be important.

The second set of viruses described in this chapter contain a single reporter gene under the control of a heterologous promoter inserted into the endogenous LAT regions after LAP1 and LAT P2. Whilst the approach of inserting a transgene directly downstream of the endogenous LAT P2 offers reduced disruption of the structural integrity of the LAT region, the capacity for expressing multiple exogenous genes is more limited. An internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) has been successfully used by our laboratory to facilitate expression of two exogenous genes from this site (Wagstaff *et al.*, 1998). An IRES has also been used to allow ribosomal access to a *lacZ* gene placed in the LAT transcript, facilitating long term expression of this reporter gene from within the endogenous HSV1 LAT region (Lachmann and Efstathiou, 1997). These two reports demonstrate the potential for directing the stable expression of multiple genes from this site. However, if IRESs of different sequence were used, this approach could also potentially be incorporated into the bicistronic cassettes, such that each cassette might drive the expression of four exogenous genes.

In this second part of the chapter, the insertions made into the LAT regions are based on the pR19 cassette. This cassette was described in section 5.2 when it was used to insert a CMV-*lacZ* cassette into the LAT region to facilitate subsequent removal of LAT P2. The reason for the use of this cassette in this chapter was again based on parallel work in our laboratory where pR19 based viruses had been shown to direct high level, long term expression of reporter genes in the PNS. The pR19 cassette and insertion site in the LAT region are very similar to those described in a previous report which showed that the CMV promoter was not capable of directing long term expression in the PNS (Lachmann *et al.*, 1996). However, Lachmann *et al.* used a truncated version of the CMV promoter (370 base pairs) whereas the pR19 cassette used in our laboratory contains a full length CMV promoter (654 base pairs). Work in our laboratory has shown that an even further truncated form of the CMV promoter (156 base pairs) is inactive during latency (Palmer *et al.*, 2000). These differences in promoter length might therefore provide an explanation for the apparent discrepancy between ours and the previously published results.

The viruses described in this chapter are all deficient in VP16, ICP34.5/ORF P, ICP27 and ICP4. As has been previously described, the rationale for this set of mutations was to attempt to minimise the expression of all of the HSV1 genes without the need to delete all the IE genes individually. The premise that this subset of deletions would be sufficient was partially based on the reported ability of the *in1814* mutation to reduce the transactivating capability of VP16. It had been shown that a virus with this mutation was reduced by at least 90% in its ability to stimulate IE gene transcription (Ace *et al.*, 1989). However, a recent report demonstrated that a virus with the *in1814* mutation in VP16 and with ICP0 inactivated was much more toxic than a virus with the entire transactivating domain of VP16 deleted and ICP0 inactivated (Mossman and Smiley, 1999). This report suggested that viruses with the *in1814* mutation may retain a residual ability to stimulate gene expression, possibly mediated through an interaction of VP16 with the tegument protein VP22. Even though this work was only very recently reported, it demonstrates the importance of characterising the expression levels of a number of HSV1 genes which have not been specifically deleted in the viruses described in this thesis. It has been reported that infection of non-permissive cells with

an ICP4 deletion mutant is associated with expression of the remaining four HSV1 IE genes and ICP6, but no other early or late genes (DeLuca *et al.*, 1985). Significant expression of ORF P (the gene which is transcribed antisense to ICP34.5) has since been observed from such viruses (Lagunoff and Roizman, 1994). However, this latter observation is of limited relevance here as all the viruses described in this chapter already have ORF P deleted along with ICP34.5. It was therefore imperative to assess the levels of ICP0, ICP22, ICP47 and ICP6 expressed from the viruses described in non-permissive cells.

The main aims of this chapter were therefore to construct stable viruses containing bicistronic expression cassettes in non-LAT sites and single expression cassettes in the endogenous LAT regions and to assess the expression capabilities and toxicity of these viruses on non-complementing cells *in vitro*.



## 6.2 The choice of *vhs* as an insertion site for the bicistronic cassettes

As a result of the genome instability problems discussed in chapter 3, the genes of interest (wild type and mutant APP and PS1) had been cloned into the bicistronic cassettes in *vhs* flanking regions and recombined into the less disabled virus, 1764 27-UL41, the gene encoding *vhs*, is far removed from the LAT region and the bicistronic reporter gene cassettes had been found to be reasonably stable in this locus in the context of the less disabled virus. These 1764 27- APP/PS1 viruses are mentioned briefly in chapter 8 but were not pursued for reasons which will be discussed in that chapter. However, since the genes of interest had already been cloned into the bicistronic cassettes in *vhs* flanking regions, it was decided to insert the reporter gene cassettes in this locus also. This would mean that when the fully disabled equivalents of the APP/PS1 viruses were constructed, the reporter gene viruses described in this chapter would provide an appropriate negative control.

The *vhs* protein is a component of the tegument and is known to cause accelerated degradation of cellular and viral mRNAs during the early stages of HSV infection (Kwong and Frenkel, 1987). Although it has been suggested that *vhs* plays a significant role in pathogenesis (Strelow and Leib, 1995), and may contribute to the toxicity of HSV1 vectors (Breakefield and DeLuca, 1991; Leib and Olivo, 1993), the contribution of this protein to the cytotoxicity of a virus deficient in ICP4 was found to be negligible in cultured fibroblasts (Johnson *et al.*, 1994). Additionally, strain 17+ is known to cause less inhibition of host cell polypeptide synthesis than some other HSV1 strains (Fenwick and Everett, 1990) so the benefit of insertionally inactivating *vhs* in the vectors described in this chapter might not be expected to be significant. However, since the viruses described here were expected to express only very low levels of any HSV1 proteins, any potential remaining toxicity might be attributable to virion components, of which *vhs* is one. Furthermore, it has been reported that in the absence of viral gene products, *vhs* inhibits reporter gene expression (Pak *et al.*, 1995). This unsurprising observation would also suggest that it might be advantageous to remove *vhs* from the vector backbone. A potential disadvantage of using this insertion site is that *vhs* is not an essential gene. As has been discussed, insertion into the locus of an essential gene ensures that if the recombinant virus undergoes homologous recombination with a wild-

type virus, the resulting transfer of the expression cassette would delete the essential gene in the wild type virus. While this situation would be preferable, it is clearly more relevant to gene therapy than to basic research applications. Inactivation of another viral gene (particularly one encoding a virion component) from an already very disabled backbone is more likely to be advantageous than disadvantageous in vector design and *vhs* does represent a truly ectopic site from which to study the potential of LAT P2 in controlling long term gene expression.

### **6.3 Insertion of bicistronic expression cassettes into the *vhs* locus of virus 1764 27- P2- 4- LU/4**

The *vhs* flanking regions had been previously constructed in the laboratory and consisted of HSV1 nucleotides 90313 to 93660 (a *KpnI-HpaI* restriction fragment) inserted into pGEM5 (Promega). Unlike the flanking regions for ICP4 and ICP27 which were described in chapter 3, the *vhs* flanking regions contain the coding sequence for the gene. Since *vhs* is non-essential for HSV1 growth, its inactivation does not need to be complemented in the cell lines used for virus propagation and thus the gene does not need to be completely deleted (as there are no possibilities for homologous recombination between the virus and the cell line). The expression cassettes were therefore inserted into a unique *NruI* site at nucleotide 91854 in the coding region, insertionally inactivating *vhs*.

The shuttle plasmids containing pR20.5 and pR20.9 in *vhs* flanking regions were linearised using a unique *XmnI* site in the vector backbone and separately cotransfected with infectious viral DNA prepared from virus 1764 27- P2- 4- LU/4, the construction of which was detailed in chapter 5. Recombinant plaques were identified by their ability to express *lacZ* and GFP and plaque purified.

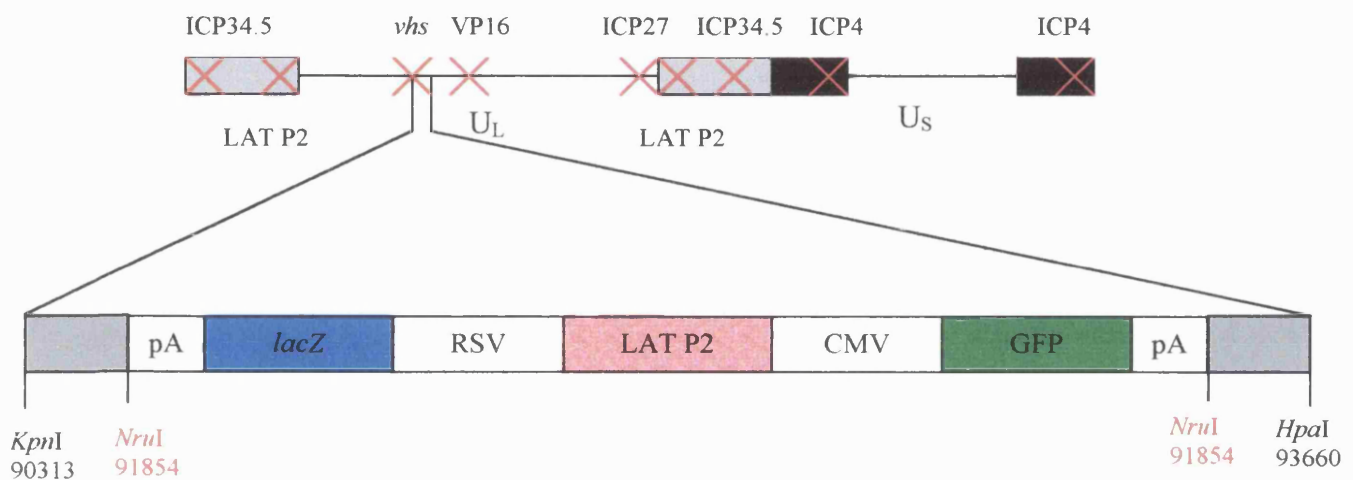
### **6.4 Virus 1764 27- P2- 4- *vhs*- pR20.5 can be stably propagated**

Following the deletion of both copies of the endogenous LAT P2 region as described in chapter 5, it was found that (as anticipated) viruses with a LAT P2 containing cassette inserted at an ectopic site could now be stably propagated. The virus 1764 27- P2- 4- *vhs*- pR20.5 was grown up on a large scale and this stock titrated on the 27/12 cell line

and the 27/12/M:4 cell line. The ability of the virus to grow on the latter but not the former cell line confirmed its ICP4 deleted status. The observation that all plaques always expressed both reporter genes confirmed that the virus was stable. A diagram of the virus 1764 27- P2- 4- *vhs*- pR20.5 is shown in figure 6.4.1. This virus is able to direct the high level, simultaneous expression of two exogenous genes on both complementing and non-complementing cells, as illustrated in figure 6.4.2

#### **6.5 Virus 1764 27- P2- 4- *vhs*- pR20.9 can not be stably propagated**

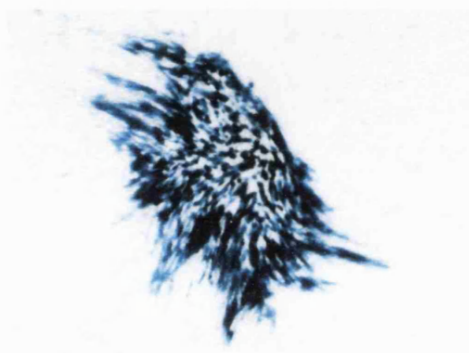
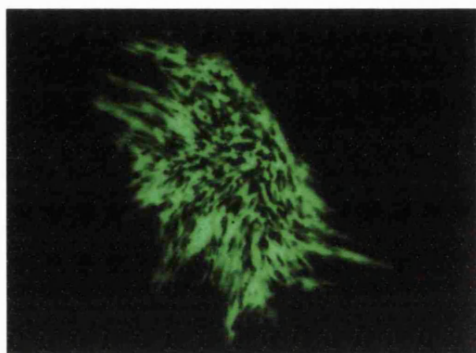
Unlike the equivalent virus containing the pR20.5 cassette, large stocks of the virus 1764 27- P2- 4- *vhs*- pR20.9/*vhs* were found to contain plaques which only contained one reporter gene. This unsurprising observation was assumed to be a result of the fact that both the endogenous LAT regions and the inserted cassette still contained the LAP1 sequence. In contrast to the situation described in chapter 3, this aberrant homologous recombination event was found to occur rarely. However, for the purposes of this thesis, work with the virus 1764 27- P2- 4- *vhs*- pR20.9 was discontinued. Future work will require the deletion of both copies of LAP1 from the endogenous LAT regions in order to make a stable background for the insertion of LAP1 containing cassettes.



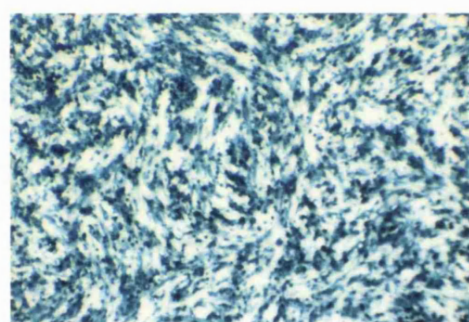
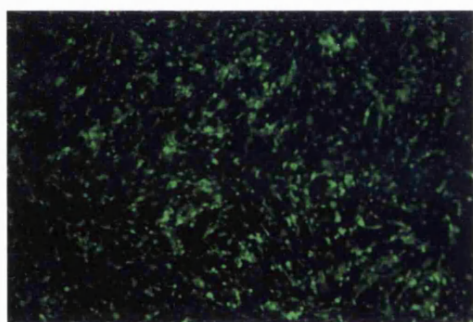
**Figure 6.4.1 The structure of the virus 1764 27- P2- 4- *vhs*- pR20.5**

A map of the virus 1764 27- P2- 4- *vhs*- pR20.5 is shown in the diagram. The insertion site is UL41, the gene encoding *vhs*. Unlike the insertions into the ICP4 or ICP27 loci, this is an insertional inactivation rather than a deletion of the gene. The *Nru*I site at HSV1 nucleotide 91854 into which the pR20.5 cassette was inserted is indicated in red. This site was destroyed in the cloning and is shown for orientation purposes only.

A)



B)



**Figure 6.4.2 Virus 1764 27- P2- 4- *vhs*- pR20.5 can direct the high level, simultaneous expression of two exogenous genes on both complementing and non-complementing cells**

A) The same plaque can be seen to be expressing *lacZ* and GFP. Cell line 27/12/M:4 was infected with virus 1764 27- P2- 4- *vhs*- pR20.5 at an MOI of 0.01. The plaque was photographed 72 hours post infection, stained for *lacZ* expression as described in section 2.3.4 and photographed again. B) Duplicate wells of non-complementing BHK cells were infected with virus 1764 27- P2- 4- *vhs*- pR20.5 at an MOI of 1. 48 hours post infection, one well was stained for *lacZ* expression as described in section 2.3.4 and the cells were photographed.

## **6.6 Expression of HSV1 genes from the virus 1764 27- P2- 4- *vhs*- pR20.5**

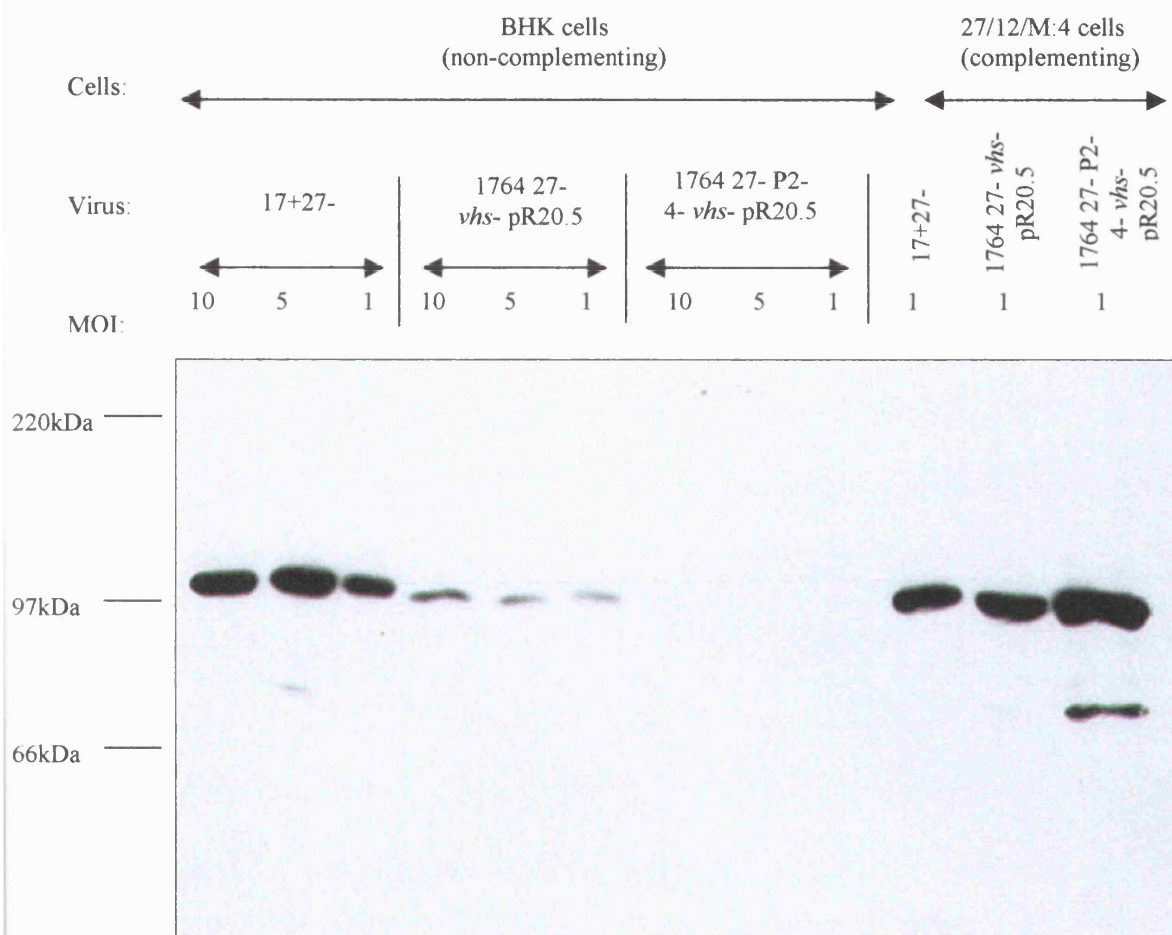
As discussed in the general introduction and in section 6.1, it might be expected that infection of non-permissive cells with an ICP4, ICP27 and ICP34.5/ORF P deletion mutant would be associated with the expression of ICP0, ICP22, ICP47 and ICP6 (DeLuca *et al.*, 1985; Johnson *et al.*, 1992). Of these four genes, ICP0 and ICP22 are known to be highly cytotoxic (Johnson *et al.*, 1994; Wu *et al.*, 1996; Samaniego *et al.*, 1997) and ICP0 is known to be a promiscuous transactivator of E and L genes (O'Hare and Hayward, 1985; Everett, 1987). The approach taken in this thesis is largely reliant on the *in1814* mutation in VP16 to minimise the expression of these genes so that the levels of their products will not be cytotoxic and will be incapable of transactivating any later HSV1 genes. The work which originally described a wild type virus with the *in1814* mutation in VP16 (Ace *et al.*, 1989) stated that the accumulation of ICP0 and ICP27 transcripts were reduced four to five fold in cells infected with this virus, but that the level of ICP22 RNA was reduced only two fold and the level of ICP4 RNA was unaffected. Non-engineered BHK cells were therefore infected at MOIs of 10, 5 and 1 with the viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5. Samples harvested at 48 hours post infection were analysed for the expression of ICP0, ICP22, ICP47 and ICP6. SDS-PAGE and western blotting was carried out as described in sections 2.5.3 and 2.5.5.

The levels of each of the proteins ICP0, ICP22, ICP47 and ICP6 expressed from the viruses described are discussed in the following sections.

## **6.7 Virus strain 1764 27- P2- 4- *vhs*- pR20.5 does not express significant levels of ICP0**

BHK cells infected with the viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5 at a range of MOIs were harvested 48 hours post infection and electrophoresed on an 8% SDS-PAGE gel. The proteins were transferred to nitrocellulose and probed with an anti-ICP0 antibody. The western blot is shown in figure 6.7.1. The figure shows that while both of the less disabled viruses expressed significant levels of ICP0 in non-complementing cells, the multiply disabled virus only

gave detectable levels of ICP0 expression after long exposure of the blot. The ICP0 expression from the 1764 27- P2- 4- *vhs*- pR20.5 virus is therefore undetectable in figure 6.7.1. Given the fact that deletion of the endogenous LAT regions would be expected to prevent the production of a transcript antisense to ICP0 and that ICP4 is known to repress the ICP0 promoter (Roizman and Sears, 1996), it was somewhat surprising to observe that the virus 1764 27- P2- 4- *vhs*- pR20.5 expresses considerably less ICP0 than virus 1764 27- *vhs*- pR20.5. This can perhaps be explained by a consideration of the time point at which these samples were taken (48 hours post infection), and the well-recognised role of ICP4 in transactivating genes of the  $\gamma$  class (Roizman and Sears, 1996; Michael *et al.*, 1988). In accordance with the essential structural role of VP16, this gene is expressed at late times post infection. The VP16 produced at this stage in the infection cycle does not normally act to transactivate IE genes at this time as a result of its interaction with *vhs*, this interaction precluding that of VP16 with its response element in IE gene promoters (Smibert *et al.*, 1994). A situation could therefore be imagined where the ICP4 produced by the virus 1764 27- *vhs*- pR20.5 is transactivating the promoter for the mutated VP16. In the absence of *vhs*, the mutated VP16 is then able to exert its residual transactivating capabilities on the ICP0 promoter, increasing ICP0 expression levels. In the case of the more disabled virus, there is no ICP4 expressed so the VP16 gene is not transactivated in this way and the ICP0 levels remain low.



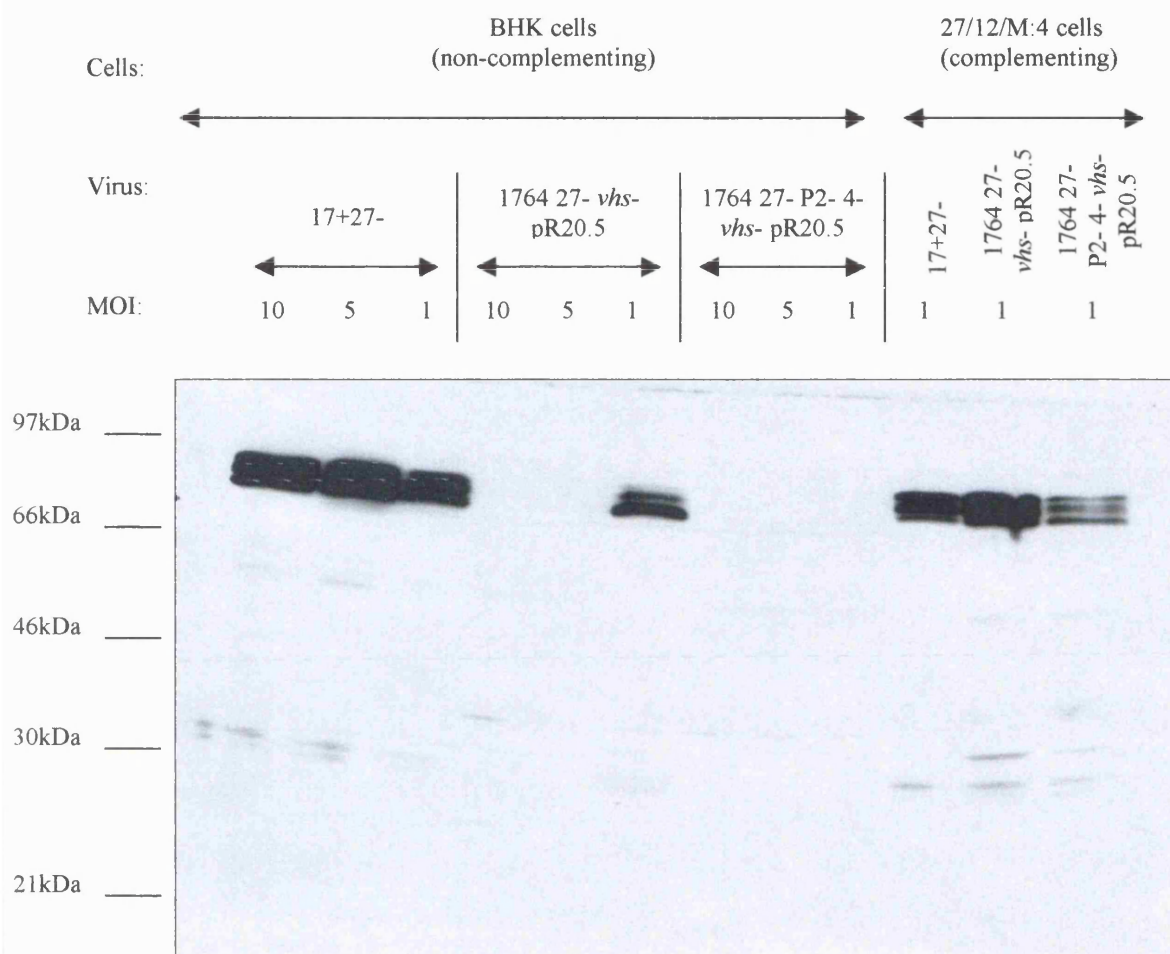
**Figure 6.7.1 Expression of ICP0 from viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5**

Non-complementing BHK cells were infected at MOIs of 10, 5 and 1 with the viruses indicated. Samples were harvested at 48 hours post infection. The samples were electrophoresed on an 8% SDS-PAGE gel followed by western blotting as described in sections 2.5.3 and 2.5.5. The blot was probed with an anti-ICP0 monoclonal antibody (purchased from Autogen Bioclear) at a 1:1000 dilution.



## **6.8 Virus strain 1764 27- P2- 4- *vhs*- pR20.5 does not express any detectable levels of ICP22**

ICP22 is non-essential for viral growth but its deletion has been found to delay the synthesis of  $\beta$  proteins and reduce the synthesis of proteins of the  $\gamma$  class (Sears *et al.*, 1985). These effects are possibly mediated through an alteration in the phosphorylation of the large subunit of cellular RNA polymerase II (Rice *et al.*, 1995). ICP22 is known to be cytotoxic (Johnson *et al.*, 1994) and it has been observed that a virus deleted for ICP27, ICP22 and ICP4 demonstrated reduced cytotoxic effects on cell structure compared to one deleted for ICP27 and ICP4 only (Wu *et al.*, 1996; Krisky *et al.*, 1998). BHK cells infected with the viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5 at a range of MOIs were harvested 48 hours post infection and electrophoresed on an 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose and probed with an anti-ICP22 antibody. This western blot is shown in figure 6.8.1. It can be seen from the figure that whilst ICP22 is abundantly expressed from the 17+ 27- virus there is little expression from the 1764 27- *vhs*- pR20.5 virus and no detectable expression from the 1764 27- P2- 4- *vhs*- pR20.5 virus, even after long exposures of the blot. Surprisingly, it can be seen that in the case of the intermediately disabled virus, 1764 27- *vhs*- pR20.5, there is greatest expression of ICP22 at an MOI of 1. This intriguing observation will be discussed further in section 6.11.

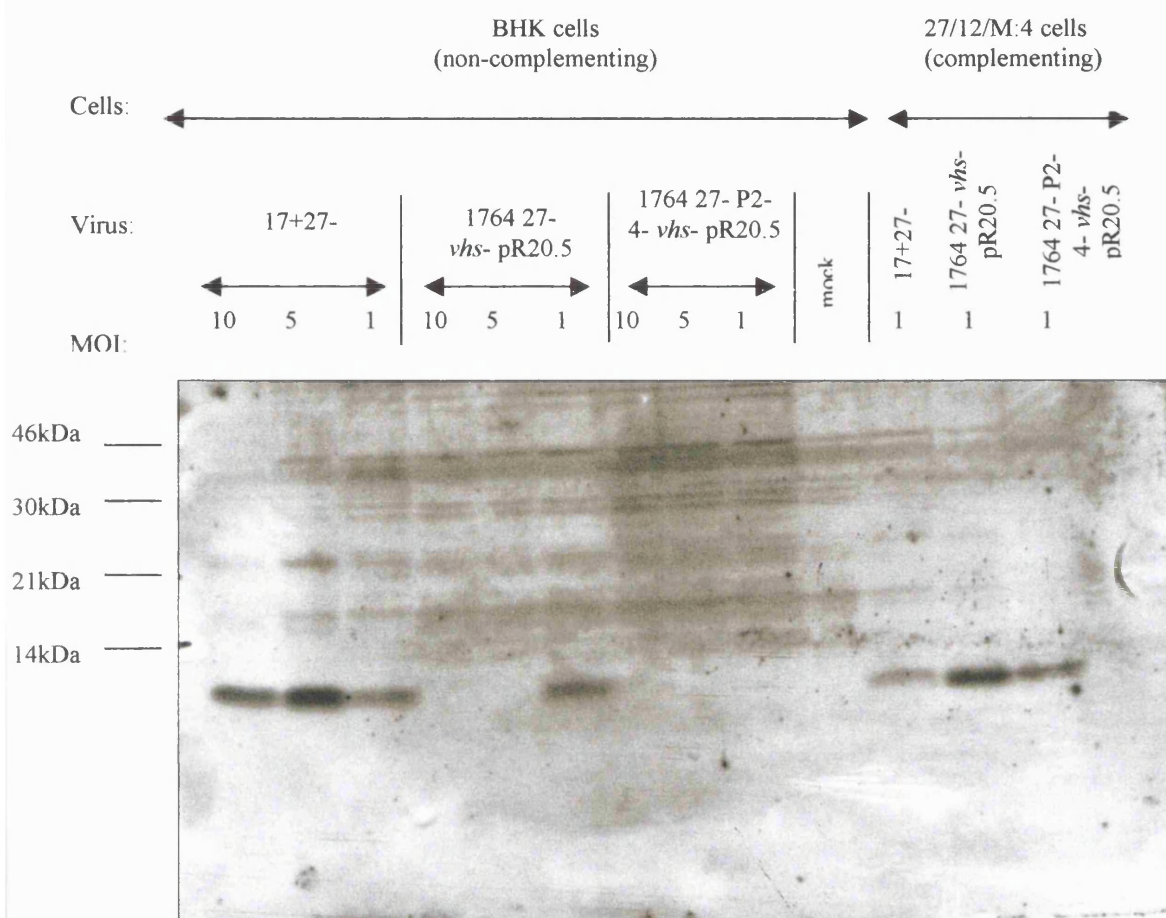


**Figure 6.8.1** Expression of ICP22 from viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5

Non-complementing BHK cells were infected at MOIs of 10, 5 and 1 with the viruses indicated. Samples were harvested at 48 hours post infection. The samples were electrophoresed on a 10% SDS-PAGE gel followed by western blotting as described in sections 2.5.3 and 2.5.5. The blot was probed with the R77 polyclonal anti-ICP22 antibody (supplied by Bernard Roizman, University of Chicago, Illinois) at a dilution of 1:500.

## **6.9 Virus strain 1764 27- P2- 4- *vhs*- pR20.5 does not express any detectable levels of ICP47**

ICP47 has been found to be non-toxic and non-essential for replication (Mavromara-Nazos *et al.*, 1986). As a result, it can be deleted from the viral genome without apparently affecting viral growth or viability. However, it is likely that the function of ICP47 is more relevant *in vivo*, where it is known to inhibit antigen presentation to CD8+ T lymphocytes, potentially providing a mechanism which allows HSV1 to escape immune surveillance by the host (York *et al.*, 1994). The promoters for ICP22 and ICP47 are identical as they are derived from sequences located within the short repeat regions of the HSV1 genome (Watson *et al.*, 1981). It might therefore be expected that the expression patterns of these two proteins would be similar. The western blot of ICP47 expression in figure 6.9.1 confirms that this is the case. However, whilst the lack of ICP47 expression from the 1764 27- P2- 4- *vhs*- pR20.5 virus is further support for the effectiveness of this set of deletions in minimising the expression of all HSV1 IE genes, it might also result in an increased susceptibility of the virus to the immune mechanisms of the host.



**Figure 6.9.1 Expression of ICP47 from viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5**

Non-complementing BHK cells were infected at MOIs of 10, 5 and 1 with the viruses indicated. Samples were harvested at 48 hours post infection. The samples were electrophoresed on a 15% SDS-PAGE gel followed by western blotting as described in sections 2.5.3 and 2.5.5. The blot was probed with an anti-ICP47 antibody (supplied by David Johnson, Oregon Health Sciences University, Portland, Oregon, U.S.A.) at a dilution of 1:500.

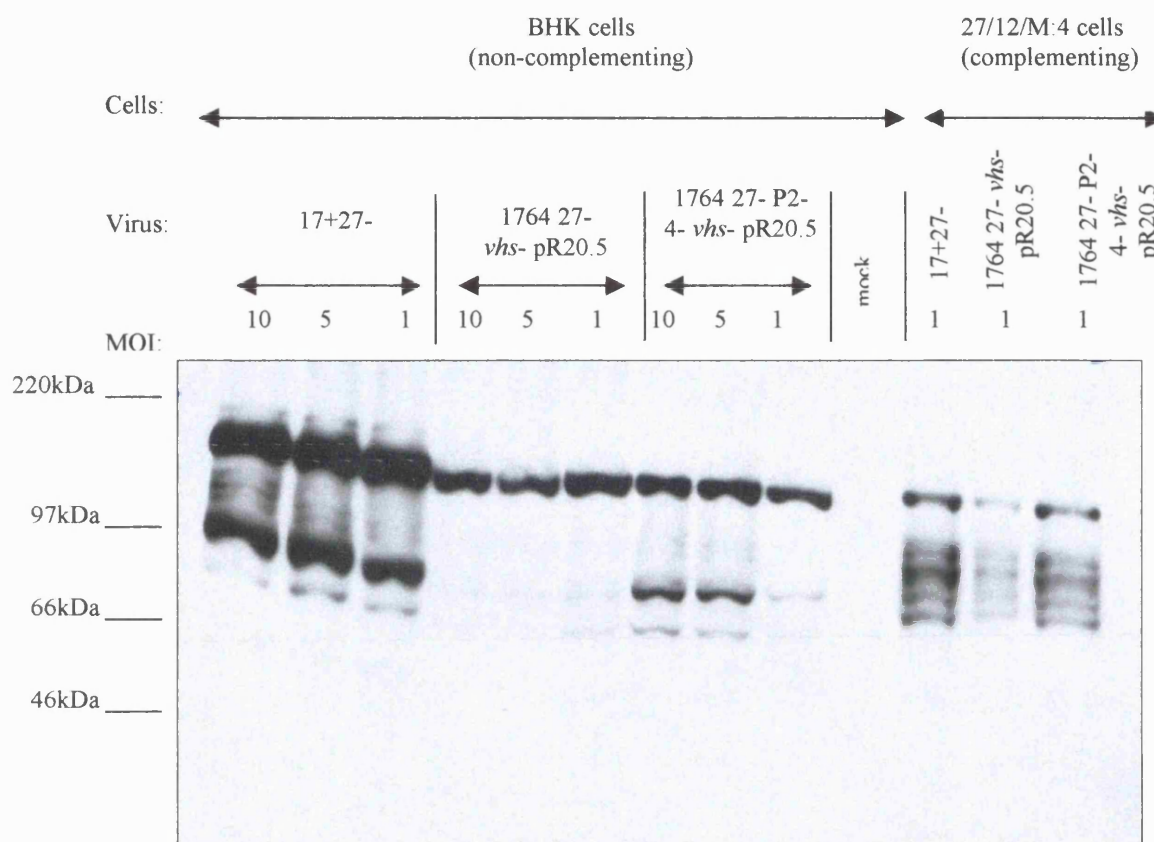
#### **6.10 The virus 1764 27- P2- 4- *vhs*- pR20.5 expresses significant amounts of ICP6**

The gene for ICP6 encodes the large subunit of ribonucleotide reductase. In accordance with the fact that it is inefficiently expressed in the absence of prior viral protein synthesis, ICP6 was classified with the E class of genes (Honess and Roizman, 1974). However, the identification of IE type *cis*-responsive elements in the promoter of ICP6 has led to it being considered as a hybrid IE/E gene (Wymer *et al.*, 1989). In support of the functional relevance of this observation, it has been shown that unlike classical E genes, high levels of ICP6 are expressed in the absence of ICP4 (DeLuca *et al.*, 1985). A western blot for ICP6 expression from the three viruses of varying levels of disablement is shown in figure 6.10.1.

It can be seen from the figure that easily detectable levels of ICP6 are expressed from all three viruses and that the deletion of ICP4 makes little difference to the levels observed. The reason for this could be that the ICP6 promoter is more sensitive to the transactivation by the low levels of ICP0 present than the ICP22/47 promoter. Alternatively, the differences in expression levels of ICP6 and ICP22/47 could be a result of the time point at which the samples were taken (48 hours post infection). In support of the first of these potential explanations, transient transfection assays have shown that the ICP6 promoter is highly sensitive to transactivation by low levels of ICP0 (Desai *et al.*, 1993). Furthermore, it has also been found that a virus deleted for ICP27 and ICP4 expressed abundant amounts of ICP6, but that the additional deletion of ICP0 caused a dramatic reduction in ICP6 expression levels such that its transcription rate mirrored that of a cellular gene (Samaniego *et al.*, 1997). Thus it seems likely that the high levels of ICP6 expression seen in figure 6.10.1 are as a result of the sensitivity of the ICP6 promoter to transactivation by ICP0.

Reassuringly, work which compared the toxicity of a number of viral gene products concluded that ICP6 was non-toxic, as measured by its inability to impair colony formation when cotransfected with a neomycin resistance plasmid followed by selection for 14 days (Johnson *et al.*, 1994). In light of this report it was hoped that the levels of ICP6 expressed by the virus 1764 27-P2-4-*vhs*-20.5 would not prove to be too problematical. However, deletion of ICP6 is known not to significantly impair titres and the protein does not need to be complemented for growth in culture (Goldstein and

Weller, 1988) suggesting that a sensible future development of virus 1764 27-P2-4-*vhs*- might be to delete the gene encoding ICP6.



**Figure 6.10.1 Expression of ICP6 from viruses 17+27-, 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5**

Non-complementing BHK cells were infected at MOIs of 10, 5 and 1 with the viruses indicated. Complementing 27/12/M:4 cells were infected at an MOI of 1 as a positive control. Samples were harvested at 48 hours post infection. The samples were electrophoresed on a 10% SDS-PAGE gel followed by western blotting as described in sections 2.5.3 and 2.5.5. The blot was probed with the R107 anti-ICP6 polyclonal antibody (supplied by Barklie Clements, Institute of Virology, Glasgow, UK) at a dilution of 1:1000.

### **6.11 Expression of ICP22/47 and of exogenous inserted genes from virus strain 1764 27- *vhs*- pR20.5 is not dose dependent in non-complementing cells**

Figures 6.8.1 and 6.9.1 demonstrated that the expression levels of ICP22 and ICP47 from virus 1764 27- *vhs*- pR20.5 were not dose dependent, the peak of expression occurring at an MOI of 1. In order to examine this further, non-complementing BHK cells were infected with either 17+ 27-, 1764 27- *vhs*- pR20.5 or 1764 27- P2- 4- *vhs*- pR20.5 over a wider range of MOIs and the samples harvested 48 hours post infection. Western blots were then probed for the expression of ICP22 as before. The results of this experiment are shown in figure 6.11.1. It can be seen from the figure that in the case of the 17+27- virus, the response is dose dependent but that with the 1764 27- *vhs*- pR20.5 virus, the peak of ICP22 expression is seen at an MOI of 0.5. No expression of ICP22 can be detected at any MOI from the 1764 27- P2- 4- *vhs*- pR20.5 virus. This phenomenon was investigated further and found to also apply to exogenous genes inserted into the 1764 27- *vhs*- pR20.5 virus but not the less disabled 17+ 27- virus or the more disabled 1764 27- P2- 4- *vhs*- pR20.5 virus. As an example of an exogenous gene, a western blot for CMV driven GFP expression from all three viruses is shown in figure 6.11.2. It can be seen from this figure that both viruses 17+27- (here containing the pR19GFP cassette) and 1764 27-P2-4-*vhs*-pR20.5 demonstrate a dose dependent pattern of GFP expression. However, in the case of virus 1764 27-*vhs*-pR20.5 the pattern of GFP expression exactly follows that of ICP22/47, with maximum expression levels being detected at an MOI of 0.5.



**Figure 6.11.1 Expression of ICP22 from virus strain 1764 27- vhs- pR20.5 is not dose dependent in non-complementing cells**

Non-complementing BHK cells were infected at MOIs of 10, 5 and 1 with the viruses indicated. Complementing 27/12/M:4 cells were infected at an MOI of 1 as a positive control. Samples were harvested at 48 hours post infection. The samples were electrophoresed on a 10% SDS-PAGE gel followed by western blotting as described in sections 2.5.3 and 2.5.5. The blot was probed with the R107 polyclonal anti-ICP22 antibody at a 1:500 dilution as before. It can be seen from the figure that in the case of the 1764 27- vhs- pR20.5 virus, the peak of ICP22 expression occurs at an MOI of 0.5. This observation is discussed in section 6.11.

BHK (non-complementing)

Cells:

Virus:

17+27-

176427-vhs-pR20.5

176427-P2-4-vhs-pR20.5

MOI:

10

5

1

0.5

0.1

0.05

0.01

10

5

1

0.5

0.1

0.05

0.01

10

5

1

0.5

0.1

0.05

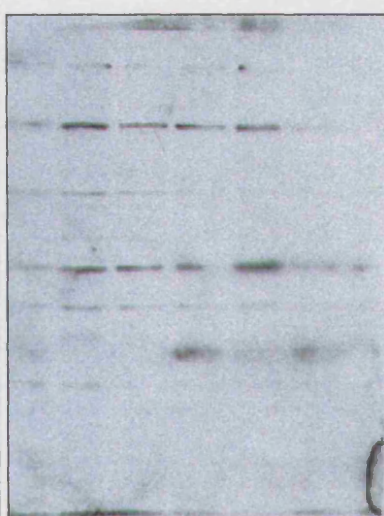
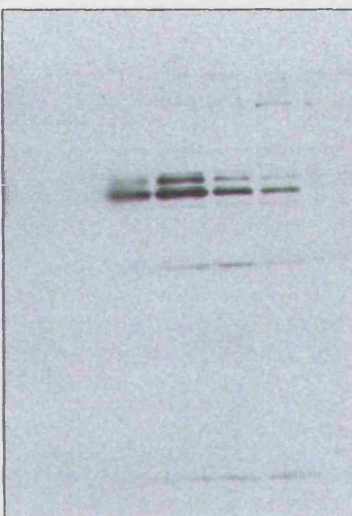
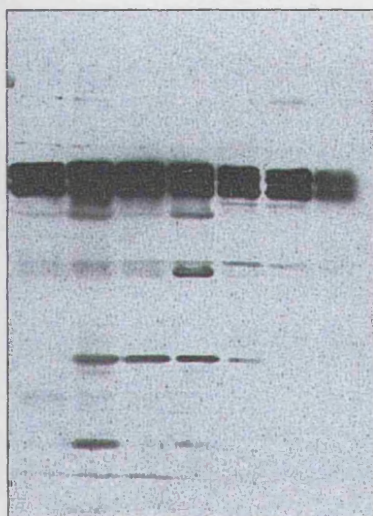
0.01

220kDa

97kDa

66kDa

46kDa



Cells:

27/12/M:4

(complementing)

Virus:

17+27-

176427-  
vhs-pR20.5

176427-P2-4-  
vhs-pR20.5

MOI:

1

1

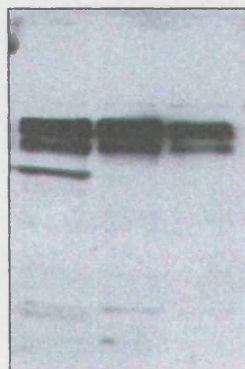
1

220kDa

97kDa

66kDa

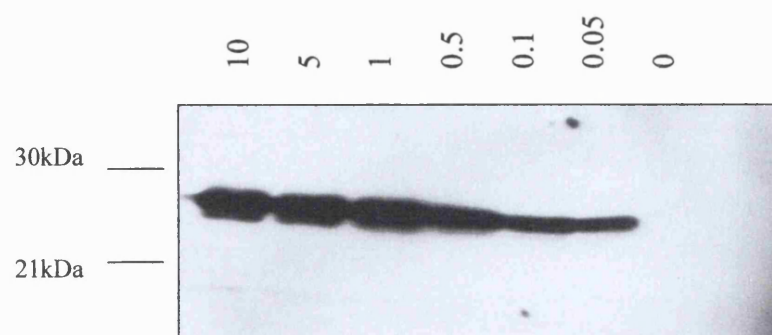
46kDa



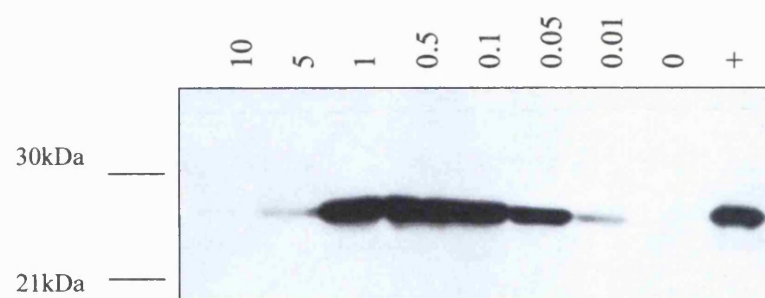
**Figure 6.11.2 Expression of an exogenous gene from virus 1764 27- *vhs*- pR20.5 is not dose dependent in non-complementing cells**

The exogenous gene is represented here by GFP. This is inserted into the *vhs* locus under the control of the CMV promoter in the pR20.5 cassette (viruses 1764 27- *vhs*- pR20.5 and 1764 27- P2- 4- *vhs*- pR20.5) or under the control of the CMV promoter in the LAT region in the pR19 cassette (virus 17+27- pR19GFP). The virus 17+27-pR19GFP was previously constructed in the laboratory and has been described elsewhere (Wagstaff *et al.*, 1998). This virus was used in this experiment because the 17+27- virus which had been used in the previous western blotting experiments (figures 6.7.1 to 6.11.1) does not contain an inserted reporter gene. Non-complementing BHK cells were infected at MOIs from 0.01 to 10 with the viruses indicated. Complementing 27/12/M:4 cells were infected at an MOI of 1 as a positive control (indicated as + in the figure). In the case of the 17+27-pR19GFP virus the positive control was run on a separate gel and is not shown. All samples were harvested at 48 hours post infection. The samples were electrophoresed on 12% SDS-PAGE gels followed by western blotting as described in sections 2.3.3 and 2.3.5. The anti-GFP antibody (purchased from Clontech) was used at a dilution of 1:1000. It can be seen from the figure that in the case of virus 1764 27- *vhs*- pR20.5, the peak of GFP expression occurs approximately at an MOI of 0.5. In the case of viruses 17+27-pR19GFP and 1764 27-P2-4-*vhs*- pR20.5, the expression of GFP is dose dependent in a linear fashion. This observation is discussed in section 6.11. The reason for the weak GFP signal in the case of the 1764 27-P2-4-*vhs*- pR20.5 virus is not known as cells infected with this virus appear to express GFP strongly when examined by fluorescence microscopy (see figure 6.4.2). However, the blots shown in this figure were not performed in parallel, possibly providing an explanation for this apparent difference.

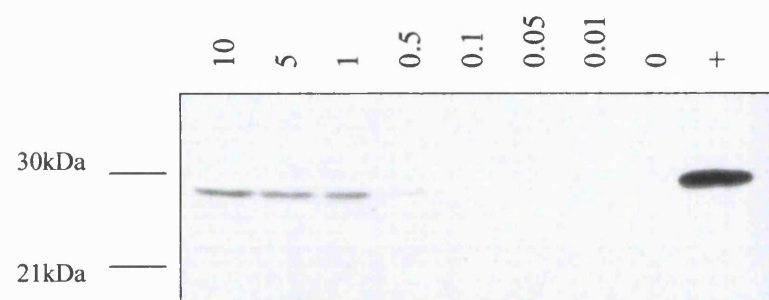
17+27-pR19GFP



1764 27- vhs-pR20.5



1764 27- P2-4-vhs-pR20.5



The reason for this unusual expression pattern is unclear but a similar observation has been reported previously in the literature (Gelman and Silverstein, 1987a; Gelman and Silverstein, 1987b). In these papers, the authors designed a series of constructs consisting of each of the regulatory IE gene promoters fused to the gene for chloramphenicolacetyltransferase (CAT). These constructs were then used in a series of transient transfection experiments in combination with plasmids encoding various viral proteins under the control of their own promoters. It was observed that ICP0 alone was a strong transactivator of all the IE gene promoters and that ICP4 was a repressor of its own promoter and that of ICP0 but a weak activator of ICP22 and ICP47. However, when plasmids encoding both ICP0 and ICP4 were cotransfected with any of the target promoter-CAT constructs, it was found that the ratio of the ICP0 + ICP4 plasmids to the target plasmids was important in determining if the target promoter was activated or repressed. When the ratio of ICP0 + ICP4 to target was low, the target promoter was activated. However, when the ratio of ICP0 + ICP4 to target was high, the target promoter was repressed. The authors described these results as a “spike response”. The possibility that the effect was attributable to promoter competition for a limited pool of transcription factors was discounted by a repeat of the experiments using a plasmid containing just the ICP4 promoter in place of the plasmid expressing ICP4. In this case the effect was not seen (Sekulovich *et al.*, 1988), indicating that both the ICP0 and ICP4 proteins must be present for the spike to occur.

It would appear from figures 6.11.1 and 6.11.2 that a similar spike response is occurring in the case of the 1764 27- *vhs*- pR20.5 virus. Based on the results of Gelman and Silverstein, a model to explain the results described in this section is proposed below. At high MOIs the levels of ICP4 and ICP0 expressed by the 1764 27- *vhs*- pR20.5 virus cause repression of the ICP22/47 and CMV promoters. However, at lower MOIs, ICP4 and ICP0 act to transactivate these promoters. A consideration of the HSV1 gene cascade reveals the probable physiological relevance of this observation. Transactivation of the IE gene promoters when the concentrations of ICP0 and ICP4 are low (i.e. immediately after infection) and then repression of the IE promoters when concentrations of ICP0 and ICP4 are high (at the end of the  $\alpha$  kinetic class) probably provides a negative feedback mechanism to regulate the expression of the IE proteins.

In the case of virus 17+27-, the presence of functional VP16 presumably overrides or masks these effects and in the case of virus 1764 27- P2- 4- *vhs*- pR20.5, the deletion of ICP4 renders any transactivation dependent on cellular factors or on the residual levels of ICP0 being expressed.

ICP0 and ICP4 are well known to have a synergistic activation effect on HSV1 promoters of the  $\beta$  and  $\gamma$  class (Everett, 1984; Everett, 1987; O'Hare and Hayward, 1985) but it appears that they do not have this effect on either  $\alpha$  promoters or on heterologous, non-HSV1 promoters (Gelman and Silverstein, 1987b; Sekulovich *et al.*, 1988). Interestingly, Gelman and Silverstein observed that the spike response affected all the  $\alpha$  gene promoters. This is in contrast to the experiments presented in this chapter where it can be clearly seen from figure 6.7.1 that ICP0 is expressed in a linear, dose dependent manner from the 1764 27- *vhs*- pR20.5 virus. The reason for this difference is unclear although the papers cited above used vero and HeLa cells whereas BHK cells were used in the experiments described here. Cell type related differences were previously found to influence the spike response of the ICP27 promoter by causing it to act predominantly as a  $\beta$  gene in HeLa cells (ICP0+ICP4 elicited a synergistic activation) but as an  $\alpha$  gene in vero cells (ICP0+ICP4 elicited a spike response) (Gelman and Silverstein, 1987).

The mechanism by which ICP0 and ICP4 act to cause this spike response on IE gene promoters is not known. Mutational analysis of the IE gene promoters have revealed that a G box sequence (GGGGG flanked by 3 to 5 base pairs of symmetry) is necessary but not sufficient for the effect (Gelman and Silverstein, 1987). More recently, ICP0 and ICP4 have been found to physically interact (Yao and Schaffer, 1994), and it is known that both proteins are capable of binding DNA and factors of the basal transcription complex (Smith *et al.*, 1993; Yao and Schaffer, 1994). However, the relevance of this observation to the mechanism by which the spike response occurs remains obscure.

The situation where a regulatory protein acts as an activator of gene expression at low concentrations but a repressor at high concentrations is not unusual. Indeed, the paradigm of all repressors, the *E.coli lac* repressor, acts in this way (Straney and Crothers, 1987). At low concentrations, the *lac* repressor protein behaves as a strong

activator as it serves to recruit transcription factors to the *lac* promoter and stabilise the transcription complex. This means that as soon as the repression is removed (in this case by addition of the inducer IPTG) the system is poised to undergo immediate transcription. It could be imagined that such a quick response might be important in regulating the classes of HSV1 gene expression. However, whether a similar situation could be occurring in the case of ICP0 and ICP4 remains to be determined.

### 6.12 The 1764 27-P2-4-*vhs*-pR20.5 virus genome persists in infected non-complementing cells

There are a number of experiments which have been carried out to assess the toxicity of viral mutants to non-complementing cells. One of the most relevant of these is to assay the ability of the virus to establish a persistent infection. It has been found that a virus individually mutated for all the IE genes (but not a less disabled virus) was capable of persisting in vero cells for at least 28 days post infection (Samaniego *et al.*, 1998). In this study the authors found that expression from a CMV-GFP cassette inserted into the ICP27 locus of this very disabled virus was silenced soon after infection but that GFP expression could be reactivated by superinfection with a virus not deleted for ICP0. This was found to occur at any time up to the end of the experiment (28 days). It was proposed that this silencing was attributable to the lack of expression of ICP0 from the initially infecting virus and that it was ICP0 which was re-stimulating GFP expression upon superinfection. In order to formally prove that this was the case, it would have been necessary to determine if a transiently transfected plasmid driving the expression of ICP0 could have the same effect.

In order to examine if the 1764 27-P2-4-*vhs*-pR20.5 virus was capable of establishing a persistent infection, a similar experiment was carried out. Vero cells were infected at an MOI of 10 and maintained in low serum at 34°C for 21 days. Vero cells were used for this experiment as they are more appropriate for long term maintenance in culture than, for example, BHKs. However, in contrast to the paper by Samaniego *et al.*, in this experiment GFP expression from the 1764 27-P2-4-*vhs*-pR20.5 virus continued without superinfection until the end of the experiment. This could be attributable to either the LAT P2 region facilitating continued expression from the CMV promoter or to the low levels of ICP0 which may be expressed from this virus. In support of the former hypothesis, it would be unlikely that ICP0 was still being expressed 21 days post infection. In an attempt to distinguish between these two possibilities, the experiment was repeated, this time including a control virus 1764 27- P2- 4- MSV/GFP, the construction of which was described in section 5.5. This virus has been previously determined to express a similar level of ICP0 to the 1764 27-P2-4-*vhs*-pR20.5 virus



(data not shown), but the MSV promoter is not positioned directly next to a LAT P2 element so expression from this promoter might be expected to be transient. Vero cells were infected at an MOI of 10 with either the 1764 27-P2-4-*vhs*-pR20.5 virus or the 1764 27- P2- 4- MSV/GFP virus. The cells were maintained in low serum at 34°C for 23 days. One day before each time point, selected wells were superinfected at an MOI of 5 with the less disabled virus 17+27- (expressing no reporter gene). The results of this experiment is shown in figure 6.12.1.

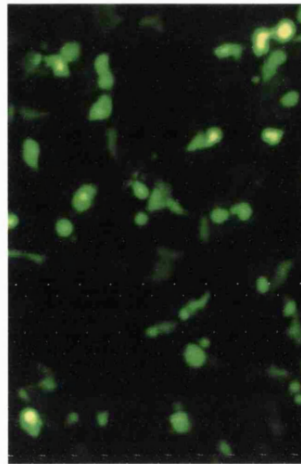
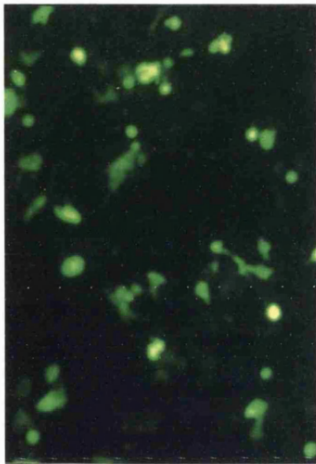
It can be seen from the figure that in the case of the 1764 27- P2- 4- MSV/GFP virus, the expression level of GFP decreases significantly over time but can be reactivated by superinfection with a less disabled virus at any time point tested up until the end of the experiment. However, the figure clearly demonstrates that this decrease in gene expression over time is considerably less marked in the case of the 1764 27-P2-4-*vhs*-pR20.5 virus. It therefore seems likely that the insertion of the LAT P2 element 5' of the CMV promoter in virus 1764 27-P2-4-*vhs*-pR20.5 is facilitating continued expression of GFP in this *in vitro* model.

This experiment demonstrates that the genomes of the multiply disabled viruses described in this thesis are capable of persisting in the nucleus of infected cells for at least 23 days. The continued expression of GFP or the ability for this GFP to be reactivated following superinfection demonstrates the viability of the infected cells and the functionality of the persisting viral genomes. Although not demonstrated here, we and others have previously found that similar experiments are not possible with less disabled viruses (for example, Johnson *et al.*, 1994). These results suggest that the viruses described in this chapter are non-toxic to vero cells in culture. In further support of this, at all times up to and including when the experiment was stopped at 23 days, the monolayers infected with either the 1764 27-P2-4-*vhs*-pR20.5 virus or the 1764 27- P2- 4- MSV/GFP virus were indistinguishable from mock infected cells.

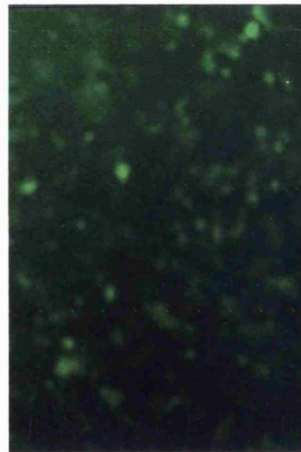
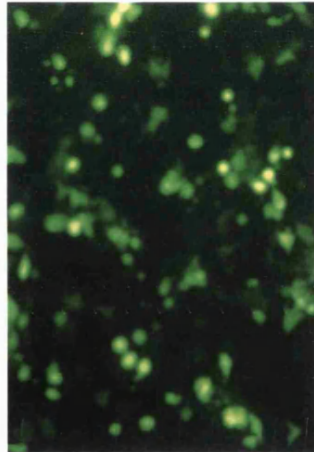
**Figure 6.12.1      Virus strain 1764 27-P2-4-*vhs*-pR20.5 can persist in cultured vero cells for at least 23 days post infection**

Vero cells at approximately 50% confluency were infected at an MOI of 10 with either the 1764 27-P2-4-*vhs*-pR20.5 virus or the 1764 27- P2- 4- MSV/GFP virus. The cells were maintained in 2% serum at 34°C for 23 days and the growth media was changed every 2-3 days. One day before each time point, selected wells were superinfected at an MOI of 5 with the less disabled virus 17+27- which expresses no reporter gene. GFP expression from the infected cells is shown. The panel on the left shows cells infected with the 1764 27- P2- 4- MSV/GFP virus and the central panel shows equivalently infected cells which have been superinfected with the 17+27- virus one day prior to the time point at which the cells were photographed. The panel on the right shows cells infected with the 1764 27-P2-4-*vhs*-pR20.5 virus in the absence of any superinfection.

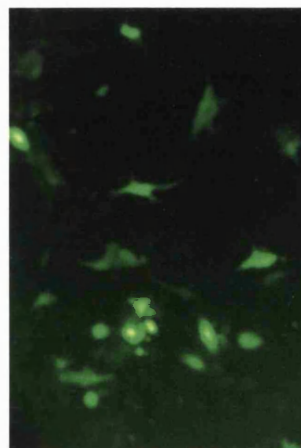
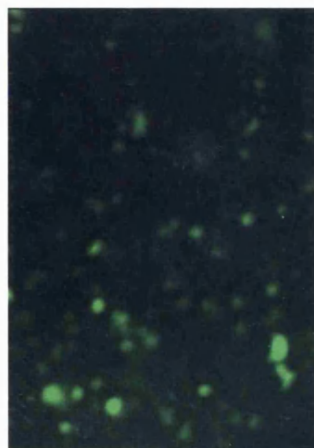
2 days p.i



7 days p.i



23 days p.i.



**1764 27-P2-4-MSV GFP**  
infection only

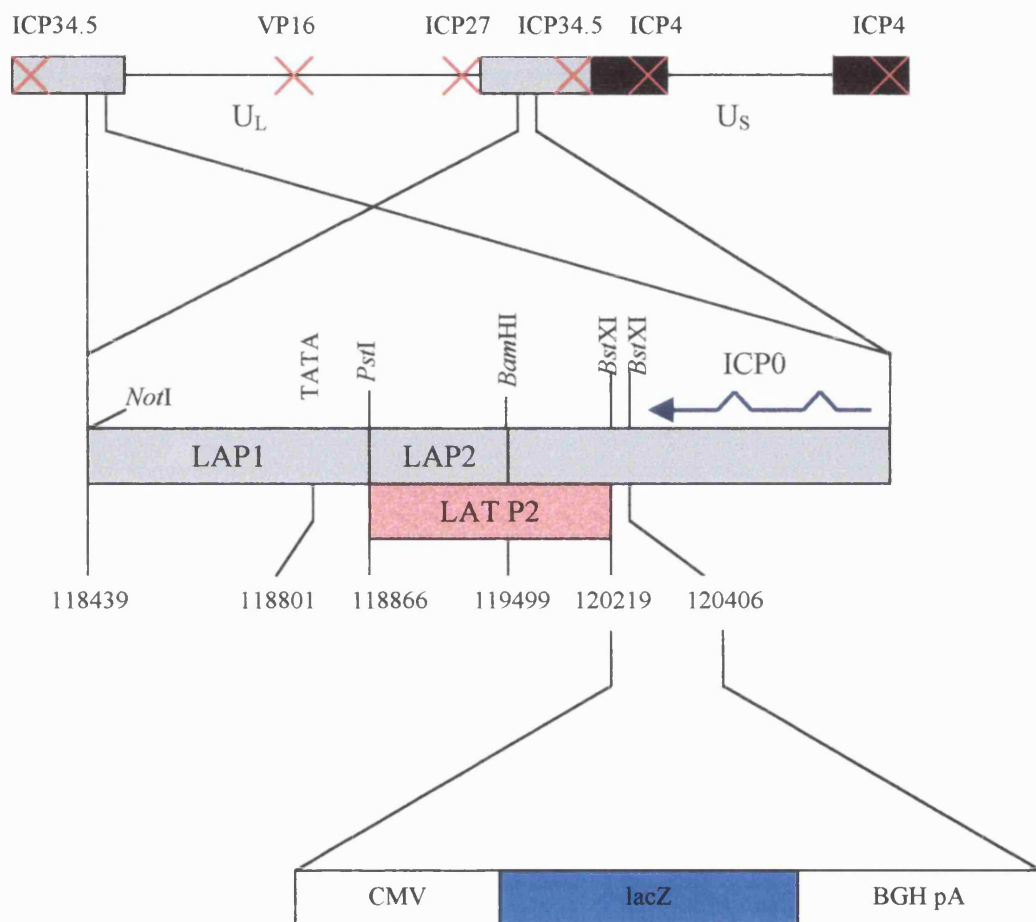
**1764 27-P2-4-MSV GFP**  
infection plus superinfection

**1764 27-P2-4-vhs-pR20.5**  
infection only

### 6.13 Insertion of expression cassettes into the LAT region of virus strain 1764 27- P2- 4- LU/4

The second part of this chapter describes the construction of viruses with single gene insertions into the endogenous LAT regions. The starting point for the construction of these viruses was again the 1764 27- P2- 4- LU/4 virus, the construction of which was described in chapter 5. However, in this case, recombination inserts LAT P2 back into its endogenous position, reconstituting the entire LAT region up to the *Bst*XI site at nucleotide 120219. It can therefore be seen that unlike the construction of viruses containing bicistronic cassettes inserted into *vhs*, the construction of the LAT P2 deleted virus was not a prerequisite for the construction of the viruses described in this section. It was used here as it provided a backbone which was deficient in VP16, ICP34.5/ORF P, ICP27 and ICP4 and also did not contain an inserted marker gene.

The plasmid which was initially used to place reporter gene cassettes under the control of the LAT elements in the endogenous LAT regions was pR19*lacZ*, which was described in section 5.2. This plasmid was linearised using a unique *Xmn*I site in the plasmid backbone and cotransfected with infectious 1764 27- P2- 4- LU/4 viral DNA. Recombinant viruses were identified by their ability to express *lacZ* and plaque purified. The resulting virus was called 1764 27- 4- pR19*lacZ* and its genome structure is shown in figure 6.13.1. An equivalent virus expressing GFP was also constructed. The plasmid pR19GFP had been previously constructed as described below. The GFP gene from pEGFPN1 (Clontech) was excised using *Hind*III and *Not*I and cloned between the *Not*I and *Hind*III sites in pcDNA3 (Invitrogen). This placed the GFP gene under the control of the CMV promoter and BGH polyadenylation signal. The CMV-GFPpA cassette was then excised from pcDNA3 using *Nru*I and *Bbs*I and inserted into pNot3.5 between the *Bst*XI sites at HSV1 nucleotides 120219 and 120406. The plasmid pNot3.5 was described in section 5.2. A map of plasmid pR19GFP is shown in figure 6.13.2A. This plasmid was also linearised using a unique *Xmn*I site in the backbone and cotransfected with infectious viral DNA, in this case prepared from virus 1764 27- 4- pR19*lacZ*. Recombinant plaques were identified by their ability to express GFP but not *lacZ* and plaque purified. The structure of the resulting virus, 1764 27- 4- pR19GFP is shown in figure 6.13.2B.



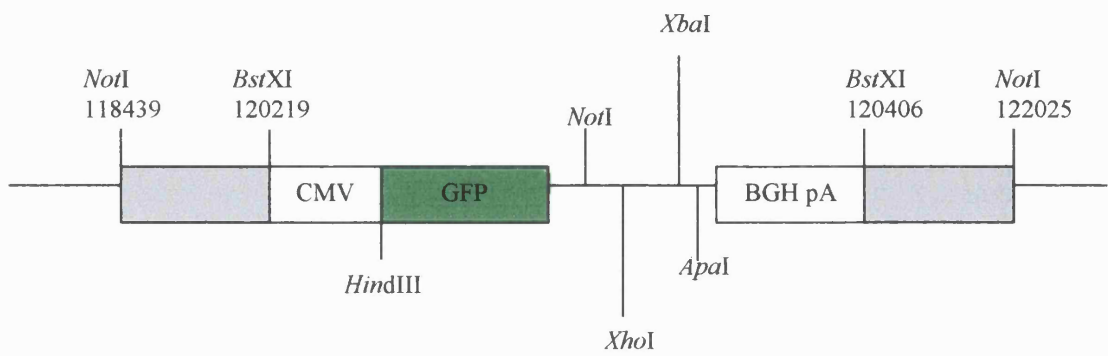
**Figure 6.13.1 Genome structure of the virus 1764 27-4-pR19lacZ**

A map of the 1764 27-4-pR19lacZ virus is shown in the figure. It can be seen that insertion of the pR19 cassette replaces the LAT P2 region which had previously been deleted from the backbone virus.

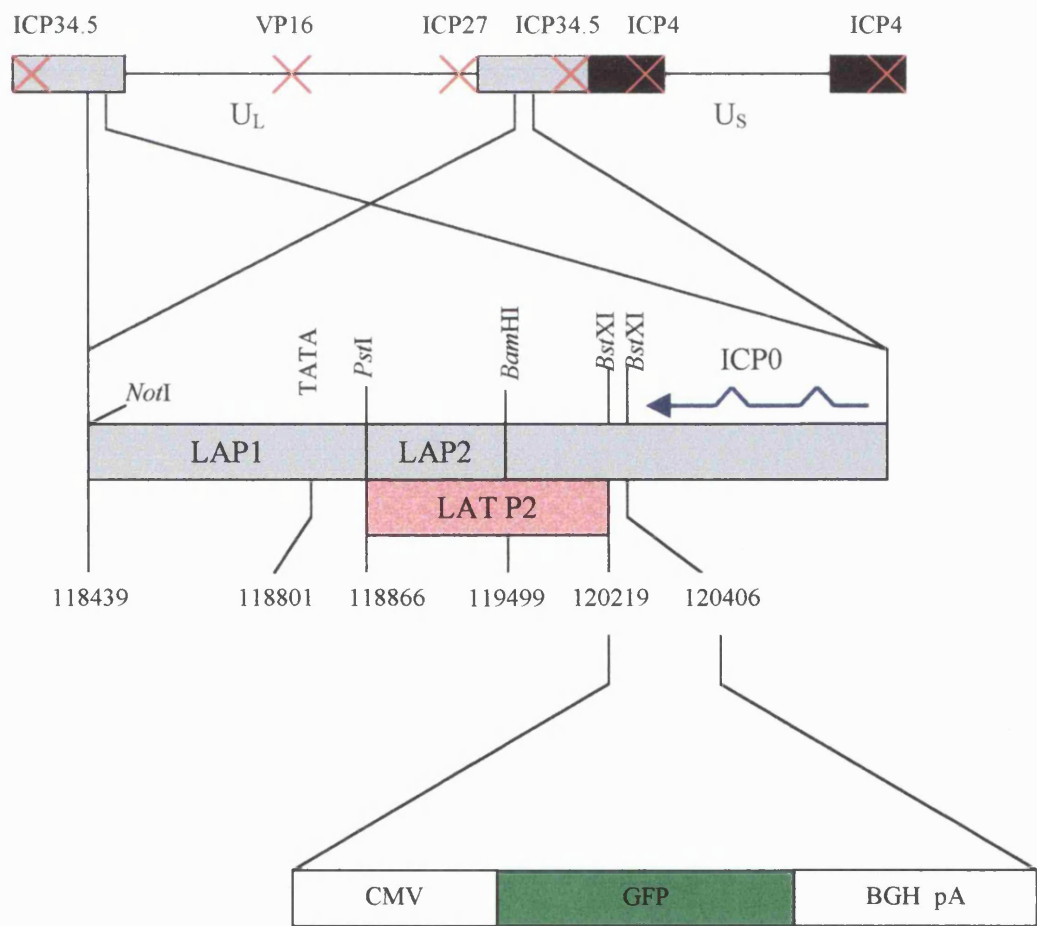
**Figure 6.13.2 Map of plasmid pR19GFP and genome structure of the virus strain 1764 27-4-pR19GFP**

A) A map of the plasmid pR19GFP is shown. The CMV-GFP cassette was cloned into LAT flanking regions as described in section 16.13. Regions homologous to the long repeats of the HSV1 genome are shown in grey and nucleotide numbers are indicated for orientation. B) The structure of virus strain 1764 27-4-pR19GFP. It can be seen that insertion of the pR19 cassette replaces the LAT P2 region which had previously been deleted from the backbone virus. This virus is exactly equivalent to the virus 1764 27-4-pR19*lacZ* shown in figure 6.13.1 but with the replacement of the *lacZ* gene with a gene encoding GFP.

A)



B)



#### **6.14 *In vitro* characterisation of viruses with insertions in the LAT region**

Viruses 1764 27- 4- pR19*lacZ* and 1764 27- 4- pR19GFP were propagated on a large scale as described in section 2.4.4. The viruses were used to infect non-complementing BHK cells and found to direct very high level expression of the respective transgene. However, it was noticed that these viruses caused low level but visible toxic effects on the non-complementing cells after several days in culture. These effects were subtle and hard to efficiently demonstrate photographically so these results are not shown. As was stated in section 6.12, this had not been the case with the viruses with inserts in ectopic sites (both viruses 1764 27-P2-4-*vhs*-pR20.5 and 1764 27- P2- 4- MSV/GFP, precluding the possibility that the difference was attributable to the presence or absence of functional *vhs*). Since the virus 1764 27- 4- pR19*lacZ* was used to make the virus 1764 27- 4- pR19GFP, the possibility that a mutation had occurred in the first virus and then been carried through to the second virus was considered. This possibility was discounted by remaking the virus 1764 27- 4- pR19GFP directly from infectious 1764 27- P2- 4- LU/4 viral DNA and observing the same slightly increased level of toxicity.

Furthermore, it had become apparent during large-scale propagation that the viruses with the inserted cassettes in the LAT region were able to be generated to higher titres on complementing cells than could the viruses with insertions in ectopic sites (quantification of this is shown in section 6.15). Neither set of viruses should be able to express any LAT antisense to ICP0 as either the LAP1 TATA box had been deleted (viruses 1764 27- P2- 4- and 1764 27- P2- 4- *vhs*-, see section 5.4) or a cassette terminated by a polyadenylation sequence had been inserted (viruses 1764 27- 4- pR19*lacZ* and 1764 27- 4- pR19GFP, see section 6.13). This was therefore discounted as a possible explanation. Parallel work was also revealing that these two sets of viruses demonstrated potential differences in their expression characteristics *in vivo* (see section 7.6). In an attempt to explain these differences, further investigations were made.

The CMV promoter is well known to be a potent enhancer of transcription, capable of functioning in either orientation to, and at some distance from, the target promoter (reviewed in Lewin, 1994). It was therefore considered a possibility that the insertion of the CMV promoter into the LAT region of virus strains 1764 27- 4-



pR19*lacZ* and 1764 27- 4- pR19GFP had facilitated enhanced transcription from nearby promoters which then resulted in elevated expression an HSV1 gene. A potential candidate promoter was that driving the expression of ICP0. Increased ICP0 expression might be expected to both enhance growth in complementing cells and increase toxicity in non-complementing cells.

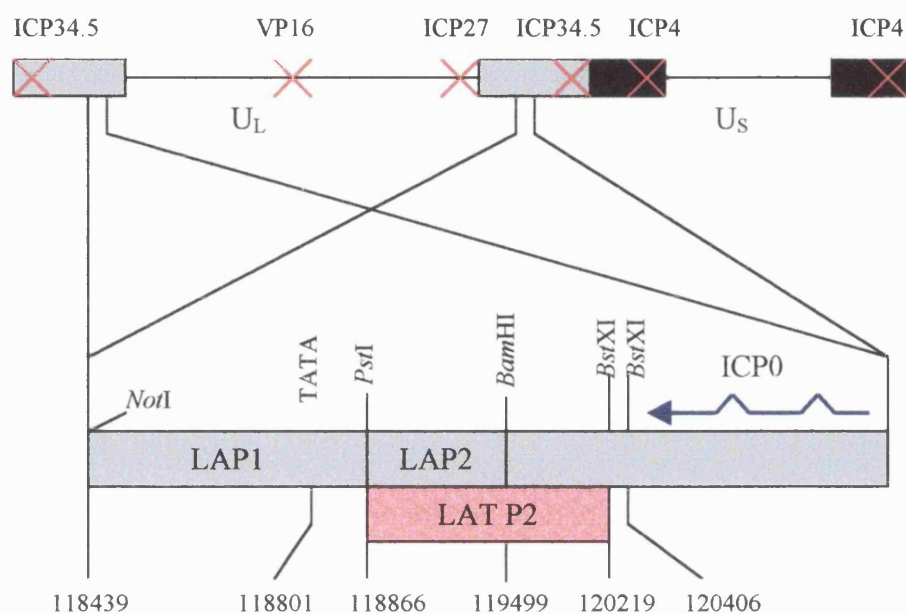
In order to identify whether the viruses with inserts in the LAT regions expressed significantly greater levels of ICP0 than similar viruses with inserts in ectopic sites, non-complementing cells were infected with a number of viruses and compared for ICP0 expression levels by western blotting. These western blots indicated that there was negligible expression of ICP0 from all the ICP4 deleted viruses, regardless of insertion site (similar to figure 5.7.1). However, this experiment may not be conclusive as the input viruses were normalised for titre, not particle number. Due to the growth differences between the viruses, it is possible that they may have different particle:pfu ratios. Although this was not measured directly, it might be assumed that the viruses with enhanced growth (i.e. those with inserts in the endogenous LAT regions) would have a lower particle:pfu ratio than the viruses with inserts in ectopic sites. Low levels of ICP0 are known to be packaged into the tegument of purified HSV1 virions (Yao and Courtney, 1992) so it may be that the number of input virions would be highly relevant to the levels of ICP0 detected. However, if the virus stocks had been normalised for particle number, then the experiment would still not provide a conclusive result as a lower infectious dose of the viruses with insertions in ectopic sites would have been used. It was therefore decided that western blotting for ICP0 levels was an inappropriate method for assessing if the CMV promoter was acting to enhance the ICP0 promoter.

Although the viruses containing insertions into the LAT region demonstrated slightly greater toxicity on non-complementing BHK cells than did the viruses with insertions into ectopic sites, this difference was not evident in cells which are naturally non-permissive for HSV1 (for example primary neuronal cultures, see section 7.2). The non-permissiveness of neurons is thought to be largely attributable to the inactivity of the HSV1 IE gene promoters, particularly the ICP0 promoter, in these cells. Therefore, although all the viruses discussed in this section express very low levels of ICP0, it is interesting to note that in situations when this expression would be expected to be even

further minimised (i.e. in neurons) the difference in toxicity between the two sets of viruses is reduced. This observation supports the hypothesis that the enhanced growth characteristics and toxicity to BHKs of the viruses containing insertions into the LAT region might be due to increased ICP0 expression.

#### **6.15 Removal of LAT P2 from the endogenous LAT regions reduces the growth of the fully disabled viruses**

In order to address the question of whether the CMV promoter was acting as an enhancer on the ICP0 or any other HSV1 promoter, and in an attempt to ascertain the mechanism by which the viruses with inserts into the LAT region replicated to higher titres, a further virus was constructed. This virus was designed to reduce the number of variables between the two sets of viruses for which the differences had been observed. A virus was therefore constructed such that it was equivalent to the virus 1764 27-P2-4-LU/4 but with an undisrupted LAT region. It would therefore be identical to the pR19-based viruses but without any inserted heterologous promoter or reporter gene. This virus was generated using the plasmid pNot3.5, the construction of which was described in section 5.2. pNot3.5 contains the *NotI* fragment from the HSV1 genome between nucleotides 118439 and 122025, encompassing much of the LAT region. This plasmid was linearised with *XmnI* and cotransfected with infectious viral DNA from the virus 1764 27- pR19GFP. Recombinant plaques were identified by their inability to express GFP. This virus was plaque purified and named 1764 27- 4- (LAT+). The structure of virus 1764 27- 4- (LAT+) is shown in figure 6.15.1. The only difference between the two viruses 1764 27-P2-4-LU/4 and 1764 27- 4- (LAT+) was therefore the absence or presence of the endogenous LAT P2 regions respectively.



**Figure 6.15.1** Genome structure of virus strain 1764 27-4-(LAT+)

A map of the virus 1764 27-4-(LAT+) is shown in the figure. It can be seen that this virus is exactly equivalent to virus 1764 27-P2-4-LU/4, with the exception that both copies of LAT P2 have been restored to their natural position.

The viruses were propagated on a large scale and it was found that virus strain 1764 27-4- (LAT+) showed enhanced growth characteristics as compared to the virus 1764 27-P2-4-LU/4. This confirmed that the growth advantage conferred on the pR19-based viruses with CMV driven inserts in the LAT regions was not due to the CMV promoter acting to enhance HSV1 gene expression. Instead, it appeared that this growth advantage was due to the presence or absence of the endogenous LAT P2 regions. The differences in growth between the viruses described are shown in the growth curves in figure 6.15.2. It can be seen from these growth curves that the viruses 1764 27- pR19-GFP and 1764 27- 4- (LAT+) grow faster and to slightly higher final titres than the viruses 1764 27-P2-4-LU/4 and 1764 27-P2-4-*vhs*-pR20.5.

The virus 1764 27- 4- (LAT+) has a complete LAT region and as such would be expected to produce LAT transcripts antisense to ICP0. However, the virus 1764 27-P2-4-LU/4 has the TATA box of LAP1 deleted (see section 5.4) so would not be expected to direct the expression of such an antisense transcript. The growth profiles of these two viruses is therefore the opposite to what might be predicted from a consideration of the likely effects of transcripts antisense to ICP0.

The fact that two copies of a 1.5kb stretch of DNA can make a difference to viral growth was surprising. The observation that 1764 27-P2-4-*vhs*-pR20.5 grew more similarly to 1764 27-P2-4-LU/4 than to 1764 27- 4- (LAT+) suggested that a copy of LAT P2 in an ectopic site did not substitute for whatever function it was performing in the LAT region. The reason for the observed differences between these sets of viruses therefore remains unknown.

A possible hypothesis is that the removal of the LAT P2 region from the 1764 27-P2-4- based viruses prevents the expression of the 2kb LAT ORF. The deregulated expression of this ORF has recently been shown to increase virus growth and, notably in this context, overcome an inactivating mutation to ICP0 (Thomas *et al.*, 1999b). Indeed, further analysis of the construct used to delete LAT P2 (pΔP2, see section 5.3) reveals that the ATG codon and first 50 bases encoding this LAT ORF have also been removed from all the LAT P2 deleted viruses used in this thesis. Whether a protein encoded by the 2kb LAT ORF is responsible for the observations of enhanced growth

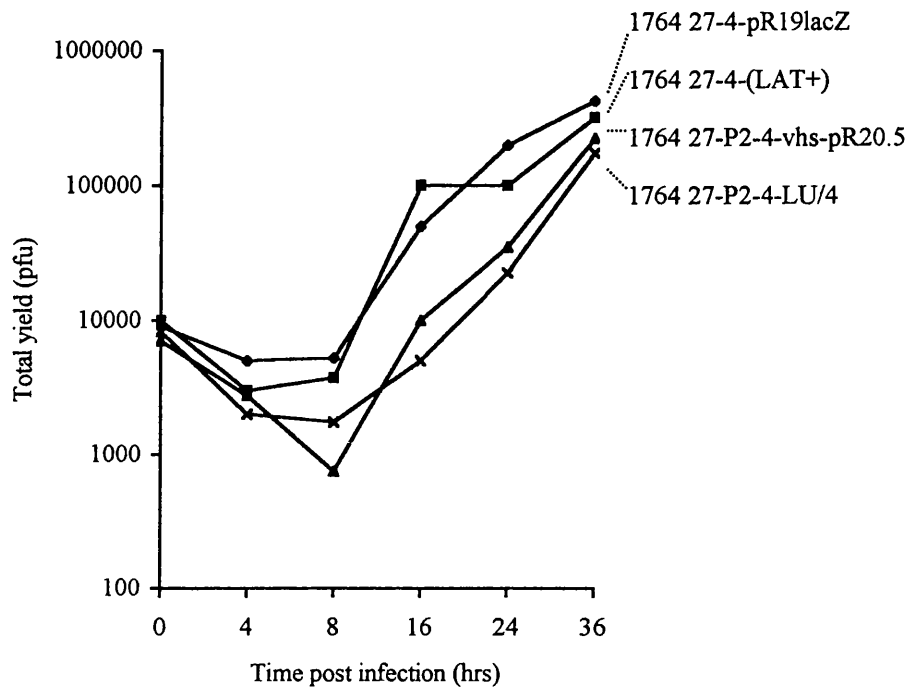
of the LAT P2 containing (and therefore LAT ORF expressing) viruses is an interesting possibility which requires future investigation.

During the writing of this thesis, a report was published which demonstrated that a region very similar to LAT P2 was capable of acting as an enhancer on the HSV1 LAP1 and *tk* promoters (Berthomme *et al.*, 2000). It is therefore possible that the LAT P2 region (rather than the CMV promoter as was originally thought) was acting as an enhancer on the ICP0 promoter when the two regions were in close proximity. It is also interesting to note that the LAT P2 sequence includes the 1.5kb region previously identified as being necessary for wild type levels of spontaneous reactivation in a rabbit model (Perng *et al.*, 1996). However, in contrast to the results described in this chapter, Perng and colleagues found that inserting the 1.5kb region of LAT DNA into an ectopic genomic locus could restore the wild type reactivation frequency. Nevertheless, it will be interesting to see if the effects observed in this chapter and those previously reported can be reconciled in a common mechanism of action for sequences within the LAT P2 region.

**Figure 6.15.2 Virus strains 1764 27-4-pR19*lacZ* and 1764 27-4-(LAT+) show enhanced growth compared to virus strains 1764 27-P2-4-LU/4 and 1764 27-P2-4-*vhs*-pR20.5**

A) Growth curves were carried out on cell line 27/12/M:4 in duplicate at an MOI of 0.01 in 24-well plates. Plaques were counted following immunostaining with a polyclonal anti-*HSV1* antibody (purchased from Dako, used at 1:400 dilution). This was to remove any bias associated with the fact that GFP or *lacZ* expressing plaques are more readily identifiable than plaques which do not express a marker gene. Yields are given as total values in pfu/well (500µl). B) Table XII summarises the main differences between the viruses used in this experiment.

### A) Growth curves of viruses with differing numbers of LAT P2 regions



**B) Table XII: Characteristics of viruses used in the growth curves**

Virus name	Deletions/ Inactivations	Insertions	Number and position of LAT P2 regions	Transcript antisense to ICP0 likely to be produced?
1764 27-4- pR19lacZ	ICP34.5/ORF P, VP16, ICP27, ICP4	CMV- <i>lacZ</i> in LAT regions	2, endogenous	no
1764 27-4- (LAT+)	ICP34.5/ORF P, VP16, ICP27, ICP4	none	2, endogenous	yes
1764 27-P2-4- LU/4	ICP34.5/ORF P, VP16, ICP27, ICP4, LAT P2	100bp luciferase fragment in ICP4	none	no
1764 27-P2-4- <i>vhs</i> -pR20.5	ICP34.5/ORF P, VP16, ICP27, ICP4, LAT P2, <i>vhs</i>	PR20.5 cassette in <i>vhs</i>	1, ectopic	no

## 6.16 Discussion

This chapter describes the construction of two sets of multiply disabled viruses, the first set having insertions in an ectopic site (UL41; the gene encoding *vhs*) and the second set containing single transgene insertions into the endogenous LAT regions. Both sets of viruses are deficient in the genes for ICP34.5/ORF P, VP16, ICP27 and ICP4 and the first set is also deficient in *vhs* and has both copies of the endogenous LAT P2 region deleted. It was found that these combinations of deletions render the viruses incapable of expressing significant amounts of any of the IE genes in non-complementing cells. This was illustrated by western blotting to detect expression of the IE genes ICP0, ICP22 and ICP47, none of which have been specifically deleted from the disabled virus.

The ICP4 deleted viruses were, however, found to express the IE/E protein, ICP6. Expression of ICP6 did not appear to cause residual toxicity as the viruses were capable of functionally persisting in vero cells for at least 23 days. This is in agreement with published reports on the negligible contribution of ICP6 to vector cytotoxicity (Johnson *et al.*, 1994).

A recent report suggested that in the context of an ICP0 deleted virus, truncation of the C-terminal transactivation domain of VP16 (the V422 mutation) provided a significantly less toxic virus than was provided by the *in1814* linker insertion (Mossman and Smiley, 1999). The authors suggested that viruses with the *in1814* mutation retained a residual ability to transactivate IE genes. It might therefore appear preferable to exchange the *in1814* mutation in the viruses described in this thesis for the V422 mutation. However, it is important to consider a difference between V422 and *in1814* which is relevant to the viruses in this thesis. The *in1814* mutation is known not to reduce the level of ICP4 at all (Ace *et al.*, 1989) whereas the V422 mutation is known to decrease the levels of ICP4 significantly (Smiley and Duncan, 1997). The comparison made in the recent paper by Mossman and Smiley is therefore essentially between a virus deficient in VP16, ICP0 and ICP4 and one deficient in VP16 and ICP0 only. The deletion of ICP0 from the virus *in1814* may have further widened the gap in ICP4 expression levels between the two viruses. It is therefore possible that the observed differences in toxicity between the viruses compared in the report by Mossman and



Smiley are largely attributable to ICP4. This might suggest that the exchange of the *in1814* mutation for the V422 mutation would make little difference in the background of a virus already deleted for ICP4. Additionally, the ICP6 promoter is thought to be only minimally transactivated by VP16 (Samaniego *et al.*, 1997), suggesting that such an exchange would not serve to significantly reduce the expression of ICP6 detected from the viruses described in this chapter.

Section 6.11 of this chapter described an interesting interaction between ICP0 and ICP4 which occurred in a virus without ICP4 deleted. These two viral proteins appeared to be interacting in an unidentified manner to repress the expression from IE and heterologous promoters at high MOIs but activate these promoters at low MOIs. However, in the more disabled virus the lack of expression of ICP4 and minimal expression of ICP0 removed these confounding factors and the expression of exogenous genes was found to be dose dependent. It might be anticipated that a dose dependent response such as that elicited by the most disabled virus would be more desirable in gene delivery applications.

A report by Samaniego *et al* in 1997 suggested that the deletion of genes from viral vectors was a play-off between reduced toxicity and compromised levels of transgene expression (Samaniego *et al.*, 1998). A series of papers by that laboratory has reported the sequential inactivation of ICP4, ICP27 and ICP22, and finally the inactivation of all the IE genes (DeLuca *et al.*, 1985; Wu *et al.*, 1996; Samaniego *et al.*, 1998 respectively). With each additional deletion the authors have reported reduced transgene expression such that in their paper describing the virus which expresses no IE genes they conclude that:

“GFP expression from the CMV promoter is highly dependent on ICP0. In the absence of all the HSV IE proteins, the level of expression was extremely low”

This observation is consistent with a report from a separate group (Preston and Nicholl, 1997). These authors observe reduced transgene expression from the CMV promoter as a result of the inactivation of VP16, ICP4 and ICP0. This report concludes that:

“promoter elements within the HSV1 genome are actively repressed in the absence of IE gene expression, and repression is not restricted specifically to HSV1 IE promoters”

In contrast to these reports, the results presented in this chapter show that although the viruses described express no significant amounts of any IE genes, the levels of transgene expression are still high. There are several possible reasons for this difference. Firstly, the viruses described here are not deleted for ICP0 and so might continue to express a minimal amount of this protein which could be serving to transactivate the heterologous promoters. If this were the case, it would be fortuitous that the ICP0 levels were low enough so that the virus was non-toxic but great enough to facilitate the continued high level expression of a transgene.

The second difference between some of the viruses described in this study and those previously reported is that none of the viruses previously described were deleted for UL41, the gene encoding *vhs*. However, the possibility that *vhs* might be contributing to the observed repression of transgene expression was not considered in any of the papers mentioned. Being a virion component, *vhs* would still be present in non-complementing cells despite the deletion or inactivation of all the IE genes. The mechanism by which *vhs* works to destabilise mRNA is indiscriminate and it has been found that *vhs* is sufficient to cause this destabilisation when it is expressed as the only HSV1 protein in an *in vitro* translation system (Jones *et al.*, 1995). It might therefore be possible that *vhs* is responsible for a destabilisation of the transgene transcripts, resulting in an apparent repression of gene expression. Support for this hypothesis is provided by a paper which stated that *vhs* could indeed decrease reporter gene expression in the absence of other viral gene products (Pak *et al.*, 1995). However, whether *vhs* could account for the reported continued absence of transgene expression many days after infection would seem very unlikely. In any case, *vhs* has only been inactivated in some of the viruses described in this chapter, although all can give high level transgene expression.

A third difference between the work in this thesis and that reported in the literature is the context of the CMV promoter. In the series of papers from the DeLuca laboratory, the CMV-GFP cassette is inserted into the ICP27 locus and in the paper by Preston and Nicholl the insertions are in the *tk* locus. It is therefore possible that the presence of the LAT P2 region immediately 5' of the CMV promoter in the viruses described in this chapter has facilitated a higher, more sustainable level of transgene

expression despite the absence of the IE gene products. It is also possible that a combination of these mechanisms are responsible for the observed high level of transgene expression from the viruses described in this chapter.

The construction of viruses with single exogenous gene inserts downstream of LAT P2 in the endogenous LAT region was described in section 6.13. These viruses were observed to show enhanced growth as compared to their LAT P2 deficient counterparts. This difference was found not to be attributable to the presence of the CMV promoter, which could have been acting as an enhancer of viral gene expression. The viruses in which the LAT P2 sequences were maintained in their natural position were also observed to be slightly more toxic to non-complementing BHK cells (but not neurons, see section 7.2) than the viruses with the endogenous LAT regions deleted. This LAT P2 mediated effect was unexpected and has yet to be explained.

This chapter has described the construction and *in vitro* characterisation of a number of stable viruses containing either two reporter genes at a non-LAT site or one reporter gene in the LAT region. It would appear from the results presented that the viruses are non-toxic and, considering other results in our laboratory using less disabled viruses in the PNS, should have the potential to express transgenes in the long term. These issues are addressed in the next chapter when these viruses are used to infect a number of different primary neuronal cultures and the rat brain *in vivo*.

## **CHAPTER 7**

# **TRANSGENE EXPRESSION IN PRIMARY NEURONAL CULTURES AND THE RAT CNS *IN VIVO***

## 7.1 Introduction

The differing tropism of each of the viruses commonly used as gene delivery systems is likely to mean that no single vector will be appropriate for all applications. Adenovirus naturally infects cells of the upper respiratory tract and so has often been proposed as a gene therapy vector for cystic fibrosis. AAV has a propensity to infect muscle cells and so might be potentially suitable for the development of a treatment for Duchenne muscular dystrophy or for the secretion of systemically active molecules such as factor VIII for haemophilia. During an HSV1 infection, the virus enters the sensory neurons at the periphery and is transported by retrograde axonal transport to the cell body in the dorsal root or trigeminal ganglia. It is this natural neurotropism which has suggested that an HSV1 based vector system might be particularly suitable for neuronal gene delivery applications.

Studies on experimental HSV1 encephalitis have suggested that the virus has a specific preference for monoaminergic neuroanatomical pathways, with the limbic cortex being particularly vulnerable (Damasio and Van Hoesen, 1985). Various routes of infection have been used (intranasal, corneal or striatal injection) but in each case the virus is seen to specifically target monoaminergic pathways (predominantly the limbic structures and raphe nuclei for the first two inoculation sites and the substantia nigra for the latter). Further support for this proposed tropism comes from the observation that monoamine turnover is specifically increased during acute HSV1 brain infections and that these changes precede the onset of encephalitis (Neeley *et al.*, 1985; Seegal and McFarland, 1988). Whether this apparent tropism is attributable to a reduced permissivity for viral entry or a restriction of gene expression in some cell types is unknown.

Of relevance to the aims of this thesis, the pathways of HSV1 tropism and the neurons selectively affected in AD overlap to a certain extent. AD specifically targets neural circuits involved in memory and cognition, predominantly the neurons of the neocortex, hippocampus, amygdala, basal forebrain cholinergic system and brainstem monoaminergic nuclei (Whitehouse *et al.*, 1982; Morrison and Hof, 1997). When the brains of aged transgenic mice overexpressing mutant APP were examined, it was found that the thalamus was almost completely spared of amyloid, despite extensive

deposition in most other brain areas (Johnson-Wood *et al.*, 1997). This suggests that there is a specificity to the amyloid deposition pattern seen in AD.

The previous chapter reported the construction and characterisation of viruses which are severely deficient in the expression of all IE genes. The minimal toxicity to target cells was demonstrated by the observation that vero cells infected at high MOI remain viable for at least 23 days in culture (see section 6.12). However, it is well known that a gene delivery system which appears promising *in vitro* can be severely compromised *in vivo* by the immune response of the host. This point is illustrated by the recent case of Jesse Gelsinger, the 18 year old man who died probably as a direct result of a strong immune response to a replication incompetent adenovirus vector used in a gene therapy clinical trial.

There were two main aims to the work described in this chapter. Firstly, to establish whether the viruses described in this thesis could deliver genes efficiently to primary neurons in culture, cells which are notoriously hard to transfect. Secondly, to analyse the pattern and longevity of transgene expression from the viral vectors *in vivo* following direct injection into the rat CNS. If a primary neuronal culture system could be reproducibly transduced at high efficiency, studies with viruses containing AD-related genes would be carried out. If long term, high level gene delivery was accomplished *in vivo*, the development of a new animal model of AD might be possible.

## **7.2 Viruses are non-toxic to primary neuronal cultures**

Section 6.12 examined the toxicity of the disabled viruses to vero cells in culture. This experiment demonstrated that functional copies of the disabled viral genomes could persist in vero cells for at least three weeks post infection, with no obvious toxicity to the cells. In order to extend these findings, the viruses were used to infect primary cultures of adult rat DRG neurons at high MOI. Neurons are much more sensitive to toxic insults than are tissue culture cells so this experiment should provide a more relevant estimation of the toxicity of the vector system.

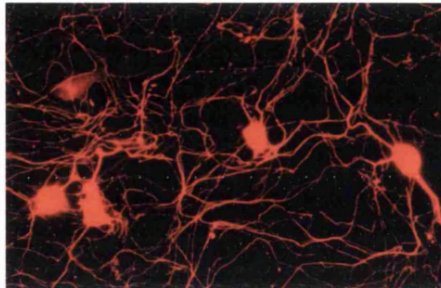
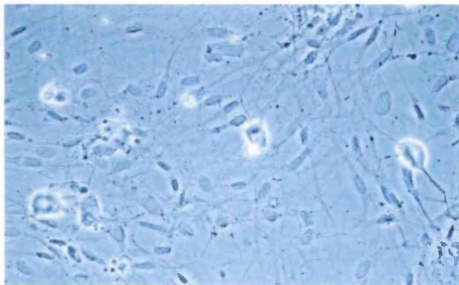
Adult rat DRG neurons were prepared as described in section 2.6.1.1 and mock infected or infected with the viruses 1764 27- P2- 4- vhs- pR20.5 or 1764 27- 4- pR19GFP at an MOI of 10. The neurons were observed to express GFP, demonstrating that they can be efficiently transduced by the disabled viruses (see section 7.3). One week post infection, the neurons were fixed in ice-cold methanol and processed for immunofluorescence with an anti neuronal specific tubulin antibody in order to allow clearer visualisation of neuronal morphology. The results are shown in figure 7.2.1. It can be seen from the figure that the infected neurons are phase bright and appear healthy. GFP expression is not shown in this figure as it has been quenched by the fixation in methanol, but is shown in figures 7.3.1 and 7.4.1 where similar transduction experiments were performed.

**Figure 7.2.1 Viruses are non-toxic to neurons in culture**

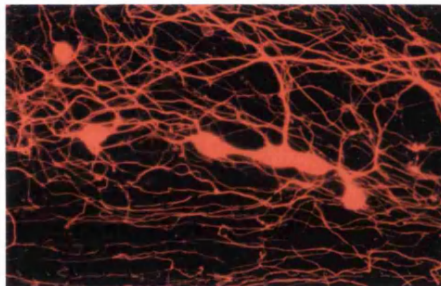
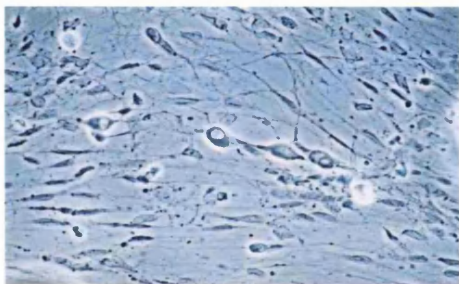
Adult rat DRG neurons prepared as described in section 2.6.1.1 were mock infected or infected with the viruses indicated at an MOI of 10. One week post infection, the neurons were fixed in ice cold methanol and stained with TUJ1, an anti neuronal specific tubulin antibody as described in section 2.5.7. This staining is shown in the panels on the right. Phase contrast photomicrographs of the same field of view are shown in the panels on the left. GFP expression is not shown in this figure as fixation in methanol quenches GFP fluorescence. An equivalent well of adult rat DRG neurons expressing GFP is shown in figure 7.3.1A.



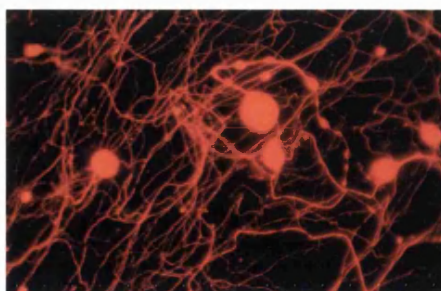
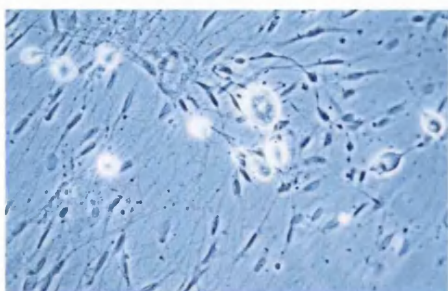
1764 27- P2- 4- *vhs*- pR20.5



1764 27- 4- pR19GFP



Mock infected



### 7.3 Neurons from different sources were infected at different efficiencies

A number of different primary neuronal culture systems were infected with the disabled viral vectors containing reporter genes in an attempt to identify the optimal conditions for infection with equivalent viruses containing genes relevant to AD. For reasons which were discussed in the general introduction, it was aimed to develop primary cultures of neurons from both rodent and non-rodent origins. The rodent neurons which were cultured were of cortical, dorsal root ganglia or hippocampal origin, taken from various embryonic and postnatal days and from either mice or rats. The non-rodent neurons were cultured from human fetuses between 12 and 20 weeks gestation. Neurons were cultured as described in section 2.6 and infected at various times post plating at an MOI of between 2 and 5. The various types of cultures used and the corresponding efficiencies of infection with the 1764 27- P2- 4- vhs- pR20.5 virus are shown in table XIII. Surprisingly, it was consistently found that certain types of primary neuronal cultures could not be infected with very high efficiency. However, it can be seen from the table that infection efficiencies could be significantly increased by extending the time in culture prior to infection. For example, the infectivity of the human fetal neurons could be improved from less than 20% to up to 95% by culturing the neurons for 17 rather than 7 days prior to infection.

Interestingly, others in our laboratory have found that mouse and rat cortical neurons infected at any time post plating could be infected at near 100% efficiency with the less disabled viruses 17+27- or 1764 (Smith *et al.*, 1998b; Wagstaff *et al.*, 1999). However, these viruses express considerably more HSV1 gene products than those described in this thesis so the level of toxicity to the neurons in these experiments would be expected to be higher. Also, the efficiencies of infection with the 1764 27- 4- pR19GFP virus appeared to be slightly higher than when the 1764 27- P2- 4- vhs- pR20.5 virus was used. The reasons for this variability in infection efficiencies is not known and possibilities are discussed in sections 7.6 and 7.7.

The three neuronal culture systems allowing reproducibly high efficiency transduction with the 1764 27- P2- 4- vhs- pR20.5 virus were selected for further use. These were adult rat DRG neurons, organotypic hippocampal slices prepared from P5 to P7 rat pups, and neurons prepared from human fetuses between 12 and 20 weeks

gestation. Examples of the gene expression following infection of these cultures with the 1764 27- P2- 4- *vhs*- pR20.5 virus are shown in figure 7.3.1. Of these, the first two systems could be reliably and reproducibly infected to high efficiencies whereas the latter was more variable. This was probably due to variation in age between the human fetuses, a factor which was not precisely known for each experiment.

Species	Age	Neuron	Type of culture	Time in culture prior to infection	Efficiency of infection
Mouse	E18 <i>cy</i>	cortical	dissociated	2 days	5%
Mouse	E18	cortical	dissociated	10 days	50%
Rat	E17	cortical	dissociated	2 days	10%
Rat	E17	cortical	dissociated	14 days	40%
Rat	E17	hippocampal	dissociated	7 days	50%
Rat	P5, P6 or P7	hippocampal	organotypic	7 days	>50%*
Rat	adult	DRG	dissociated	7 days	95%
Human	10-20 weeks gestation	mainly cortical	dissociated	7 days	<20%
Human	10-20 weeks gestation	mainly cortical	dissociated	17 days	40-95%

**Table XIII Transduction efficiency of various neuronal cultures infected with the 1764 27- P2- 4- vhs- pR20.5 virus**

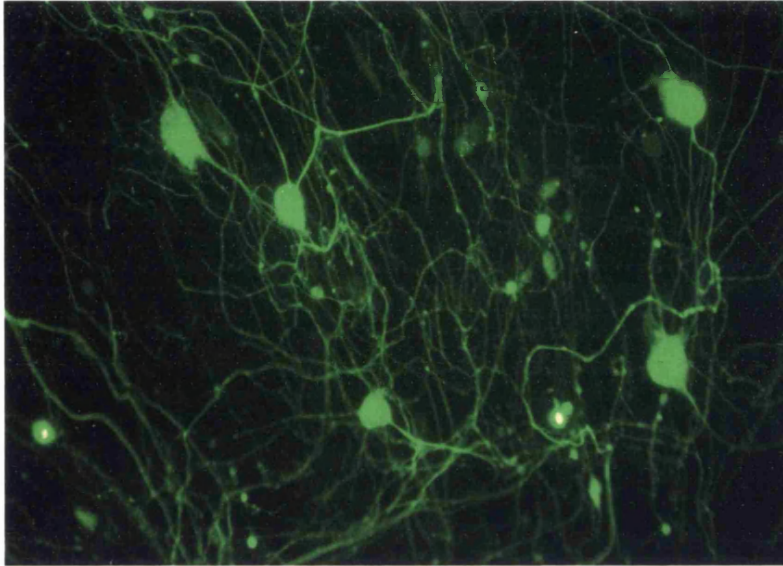
Neuronal cultures were prepared as described in section 2.6 and infected at various times post plating at an MOI of between 2 and 5. The neurons were visualised 24-48 hours post infection and scored for approximate percentage of transduced cells.

\* hippocampal organotypic cultures were infected at  $1 \times 10^6$  pfu per well (see section 2.6.2.2). The approximate infection efficiency quoted refers only to the surface layer of neurons.

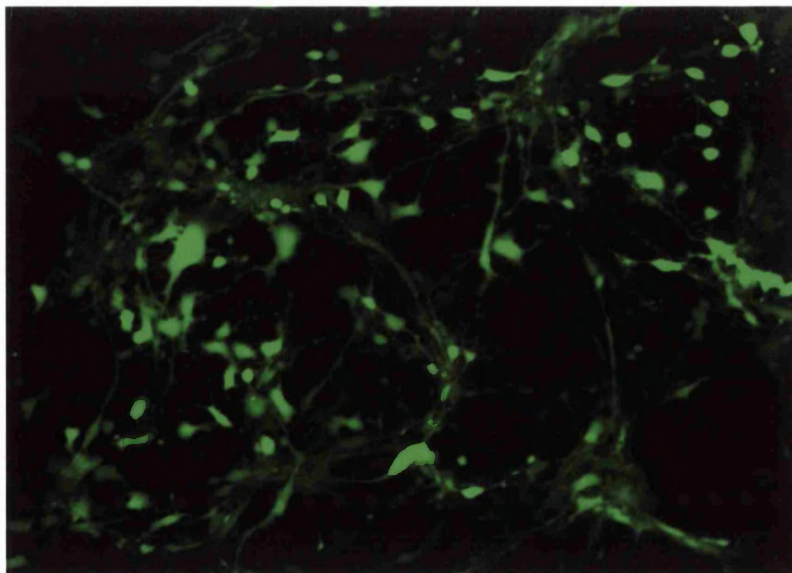
**Figure 7.3.1 Infection of primary neuronal cultures with the 1764 27- P2- 4- vhs-pR20.5 virus**

Transduction of the three most readily infected culture systems are shown. A) Adult rat DRG neurons infected at an MOI of 2 after 7 days in culture and photographed 48 hours post infection. Expression of GFP is shown. B) Human fetal neurons infected at an MOI of 5 after 17 days in culture and photographed 48 hours post infection. GFP expression is shown. C) The cellular organisation of the hippocampus is shown. The principal hippocampal region is shown in grey. The hippocampus, as seen in transverse section, has three major areas, CA1, CA2 and CA3 (CA = commissural associated regions). Pyramidal neurons of the CA region are shown in red with dendrites extending into the molecular and polymorphic layers. The molecular layer, which consists of axons and dendrites, is the white region between the dentate gyrus (DG) and the CA regions. Small black arrows indicate loop connections formed by mossy fibres and Schaffer collaterals. CN is the tail of the caudate nucleus; ENT is the entorhinal cortex; Fx is the fimbria; h is the hilus of dentate gyrus; SU is the subiculum. The dotted rectangle indicates the areas of the hippocampus present in the organotypic slice cultures. An organotypic hippocampal slice culture prepared from a P7 rat pup infected after 7 days in culture with  $1 \times 10^6$  pfu of virus and photographed 48 hours post infection is shown. The panel on the left shows *lacZ* staining from the 1764 27- P2- 4- vhs- 20.5 virus. It can be seen that expression from the virus is predominantly localised to the neuronal layers (the CA regions) of the slice. GFP expression in the region indicated by the black square is shown at higher magnification in the panel on the right. The cellular organisation of the hippocampus diagram is taken from Kiernan, 1998.

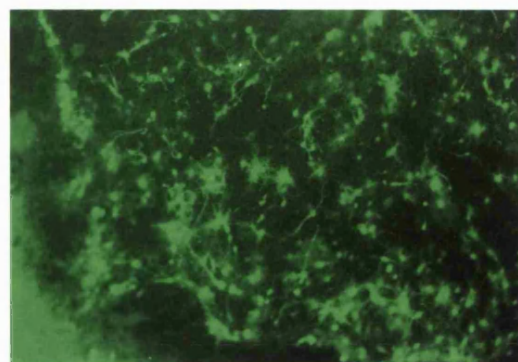
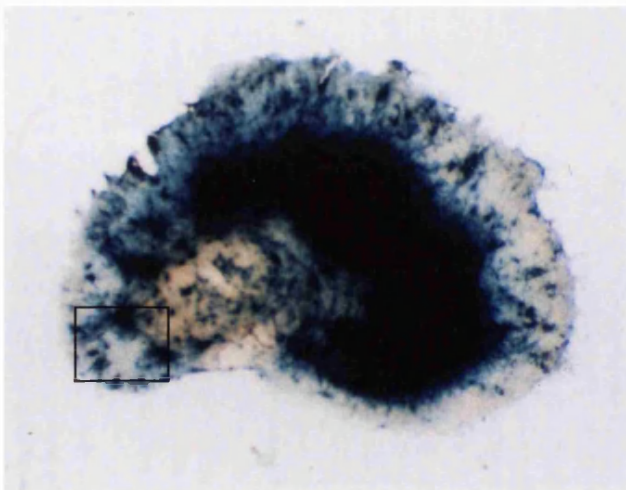
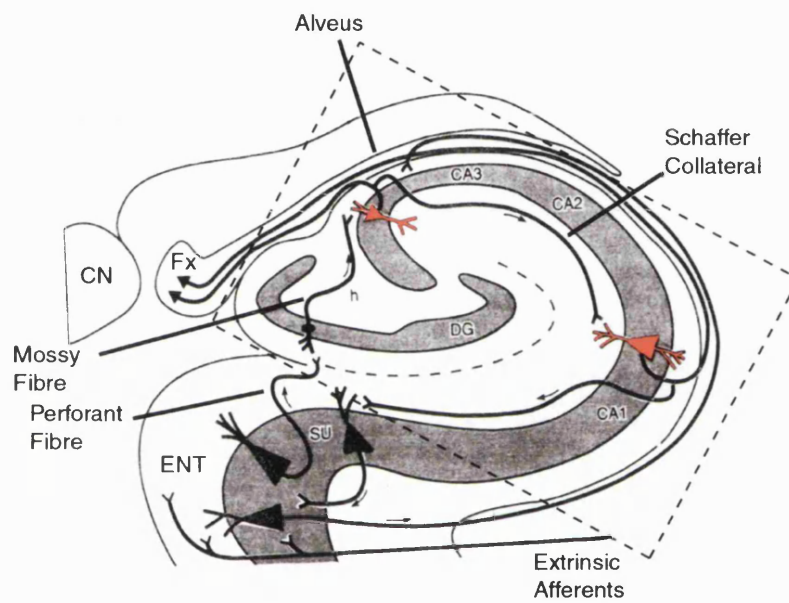
**A) Adult rat DRG neurons**



**B) Human fetal neurons**



### C) Organotypic hippocampal slice culture





#### **7.4 Increased efficiency of infection at late times post plating is not due to infection of supporting cells**

None of the cultures described were treated with cytosine arabinoside (AraC) or equivalent anti-mitotic compounds, so it would be expected that supporting cells would be present in these cultures. The cultures which could be reproducibly infected to the highest efficiencies (adult rat DRG neurons and P5-P7 rat organotypic hippocampal slices) were also those which would be expected to have the highest numbers of supporting cells. The possibility that the apparent increased efficiency of infection in these cultures, and at longer times post plating in other culture systems, was actually due to infection of an increased number of supporting cells rather than neurons was therefore considered.

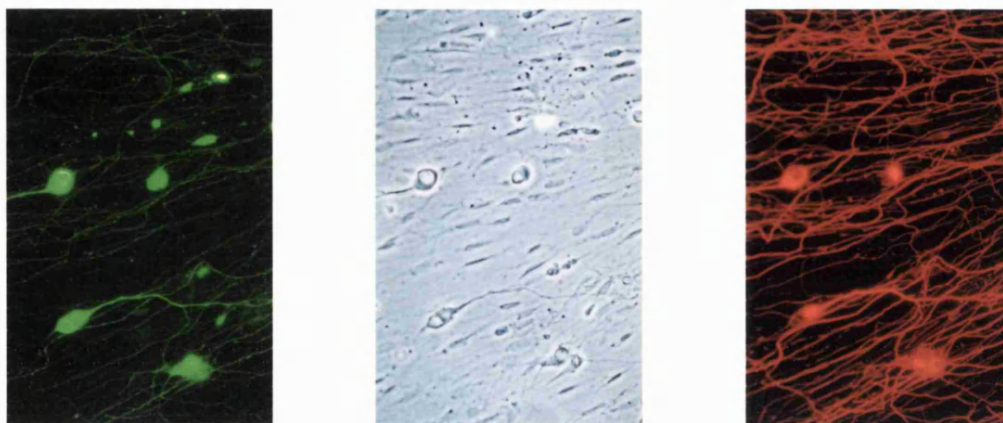
DRG neurons from an adult rat were prepared as described in section 2.6.1.1 and infected 7 days post plating with the 1764 27- P2- 4- *vhs*- pR20.5 virus at an MOI of 1. 48 hours post infection, the cells were fixed with 4% paraformaldehyde so as not to quench the GFP expressed from the virus. Immunofluorescence using TUJ1, an anti neuronal specific tubulin antibody, was carried out as described in section 2.5.7. The results are shown in figure 7.4.1. It can be seen from the figure that the TUJ1 staining and the GFP expression from the virus co-localise, demonstrating that the observed transgene expression is predominantly due to infection of neurons and not of supporting cells. Indeed it appears that the neurons in the culture are preferentially transduced, although the reason for this has not been identified.



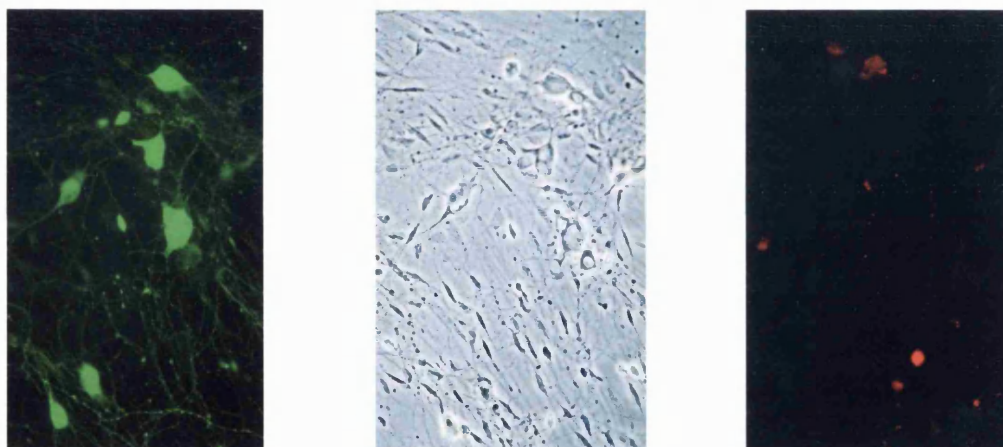
**Figure 7.4.1 Co-localisation of GFP expression and TUJ1 staining**

A) Adult rat DRG neurons infected with the 1764 27- P2- 4- vhs- pR20.5 virus at an MOI of 1 were fixed in paraformaldehyde 48 hours post infection and processed for immunofluorescence with an anti-neuronal specific tubulin antibody, TUJ1. B) Negative control infected with virus and processed for immunofluorescence as before but in the absence of the TUJ1 primary antibody. C) Negative control not infected with virus but stained with TUJ1. In each case the panel on the left shows fluorescence under fluorescein optics, the panel on the right shows fluorescence under rhodamine optics, and the central panel shows the cells visualised by phase contrast microscopy. It can be seen that in panels B and C, there is some limited fluorescence detected under rhodamine or fluorescein optics in the absence of TUJ1 or virus, respectively. However, the photographs of these controls were overexposed compared to the others and it is assumed that the low level of fluorescence detectable is either background or “bleedthrough” between filters.

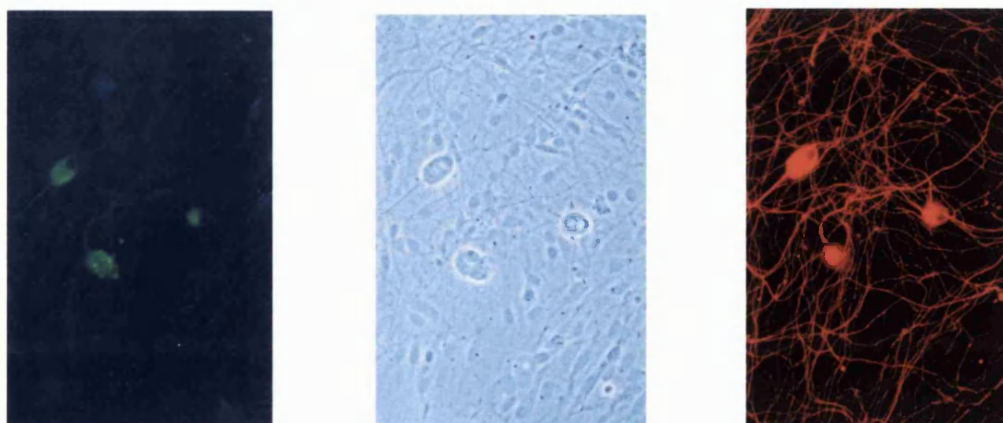
**A) Neurons infected and processed for immunofluorescence**



**B) Neurons infected but no primary antibody added**



**C) Mock infected neurons processed for immunofluorescence only**



## **7.5 Transgene expression is maintained for at least three weeks in cultured neurons**

Experiments described in section 6.12 demonstrated that transgene expression from the virus 1764 27- P2- 4- *vhs*- pR20.5 could be maintained for at least three weeks in vero cells in culture. This long term expression was attributed to the presence of the LAT P2 element as an equivalently disabled virus without this region did not afford long term transgene expression, although the viral genome persisted and transgene expression could be reactivated. In order to extend these findings, the possibility for long term transgene expression in the more physiologically relevant organotypic hippocampal slice cultures was investigated.

Organotypic hippocampal slice cultures were prepared and infected as described in section 2.6.2. At various times post infection, an insert was removed and a single slice was photographed under a fluorescence microscope. The results are shown in figure 7.5.1.

It can be seen from the figure that the high level of transgene expression observed at 2 days post infection is maintained at the 1 week time point and is still clearly detectable in the cultures at 3 weeks post infection. In this culture, a large neuron with long processes is clearly visible in the centre of the field of view (shown as an enlargement in figure 7.5.1C). It can be seen that the processes of this neuron are not swollen or beaded, indicating that the neuron has not degenerated. The infected cultures maintained normal morphology and did not differ from mock-infected control wells that had been cultured for an equivalent length of time. This again suggests that the toxic effects of this multiply disabled virus are minimal, even at long times post infection.

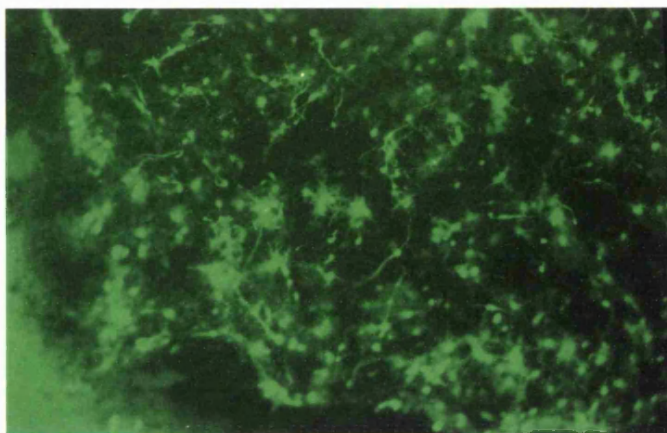
It should be noted that it is a possibility that the transgene expression observed at these long time points is not actually a result of continued expression from the viral genome but is a reflection of the stability of the proteins GFP and  $\beta$ -galactosidase. However, when vero cells were infected with a virus without a LAT P2 region adjacent to the promoter driving the transgene, high levels of GFP could only be observed up until 2 days post infection. Expression levels were then observed to reduce until very little could be detected at 3 weeks post infection but transgene expression could be reactivated by superinfection with a less disabled virus (see section 6.12). This

observation suggests that at least some *de novo* transgene protein synthesis is occurring at late times post infection in cells infected with the 1764 27- P2- 4- *vhs*- pR20.5 virus. To formally discount the possibility that the stability of the reporter gene products accounts for the apparent long term expression observed in this culture system, infection with an equivalently disabled virus without the LAT P2 region should have been included as a control in this experiment.

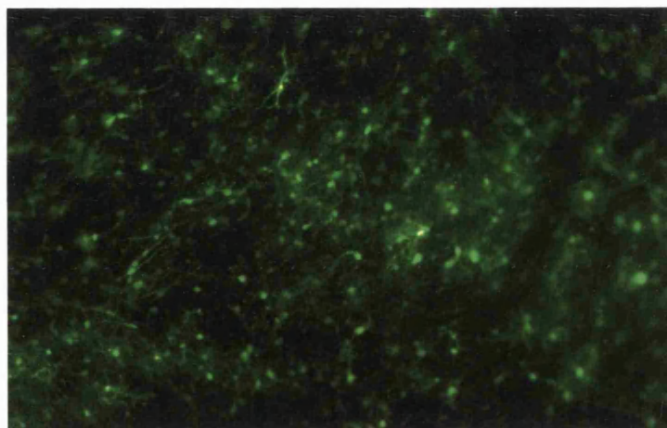
**Figure 7.5.1 Transgene expression from 1764 27- P2- 4- vhs- pR20.5 is maintained for at least 3 weeks in organotypic hippocampal slice cultures**

Organotypic hippocampal slice cultures were prepared as described in section 2.6.2.1. Slices were infected 7 days later with  $1 \times 10^6$  pfu of the 1764 27- P2- 4- vhs- pR20.5 virus. A) Expression at 2 days post infection B) Expression at 1 week post infection C) Expression at 3 weeks post infection. The region indicated by the white box is shown at higher magnification where the morphology of an infected neuron can be seen.

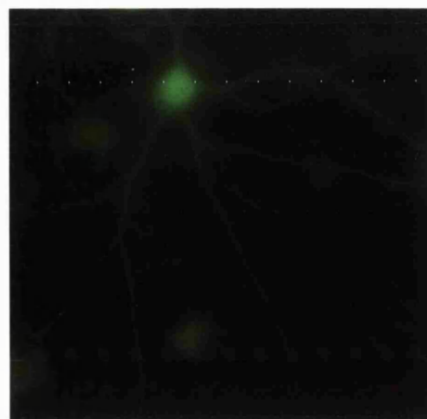
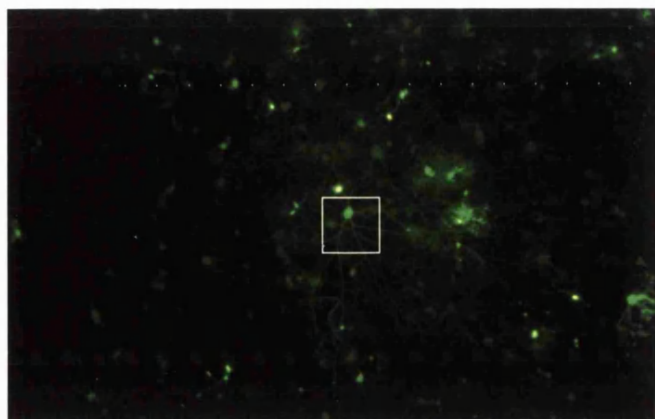
**A) 2 days post infection**



**B) 1 week post infection**



**C) 3 weeks post infection**



## 7.6 Transgene expression in the rat CNS *in vivo*

In order to examine the capacity of the viruses described in this thesis for long term expression *in vivo*, 220g female Lewis rats were stereotactically injected with  $2.5 \times 10^5$  pfu of virus in the striatum as described in section 2.6.3.1. The animal surgery was carried out by Zi-Qun Han, UCL. The rats were perfusion fixed at various time points post injection and the brains processed as described in section 2.6.3.2. 200µm slices were cut in the parasagittal plane to enable visualisation of the striatum and the substantia nigra in the same section. Less disabled viruses (17+ 27- and 1764 27-) containing the same transgene cassette were also injected as controls. The results of injection of this set of viruses, each containing the pR19*lacZ* cassette, are shown in figure 7.6.1. It can be seen that *lacZ* staining is evident not only at the site of injection but also at connected sites within the brain. This is most clearly demonstrated by transport from the striatum (injection site, indicated by a single asterisk) to the substantia nigra (indicated by a double asterisk). It can be seen from the figure that all three viruses show *lacZ* staining at the early time points but that the expression patterns are very different. These differences will be discussed in section 7.7. It can also be seen from figure 7.6.1 that the level of *lacZ* expression from the viruses 17+ 27- pR19*lacZ* and 1764 27- pR19*lacZ* is dramatically reduced between 3 days and 1 week and is almost undetectable at 1 month. However, the expression afforded by the 1764 27-4- pR19*lacZ* virus is very strong at 1 week and is still clearly evident at 1 month post injection, both in the striatum and the substantia nigra.

*In vivo* experiments with viruses containing the pR20.5 cassette are currently being carried out by others in the laboratory. Preliminary results suggest that *in vivo* levels of transgene expression from the 1764 27- P2- 4- *vhs*- pR20.5 virus are lower and less widespread than from the 1764 27-4-pR19*lacZ* virus. The reason for this is not known, particularly as the 1764 27- P2- 4- *vhs*- pR20.5 virus is very effective for long term gene delivery to cells in culture. However, it is noteworthy that following sciatic nerve or footpad injection in the PNS, higher long term gene expression in DRGs has been shown with less disabled viruses with the pR19 cassette as compared to the equivalent viruses with the pR20.5 cassette (Palmer *et al.*, 2000). It was for these reasons that the experiments described in this section were initially carried out with the

pR19 set of viruses. Whether these differences are an extrapolation of the observation made in section 7.3 that the 1764 27- P2- 4- *vhs*- pR20.5 virus appears to infect certain types of neurons in culture less efficiently than the 1764 27-4-pR19*lacZ* virus is not known. It is also possible that this difference is related to the fact that the 1764 27-4-pR19*lacZ* virus grows better in culture than the 1764 27- P2- 4- *vhs*- pR20.5 virus. For example, in the case of CNS neurons, it could be that a certain threshold level of ICP0 expression is required for efficient transgene expression and that this level is attained in the case of the 1764 27-4-pR19 virus but not in the case of the 1764 27- P2- 4- *vhs*- pR20.5 virus. These possibilities were discussed in section 6.16. Alternatively, it is possible that regions upstream of LAT P2 (within LAP1) are also required for optimal long term transgene expression *in vivo*. These regions would be maintained in the case of the 1764 27-4-pR19 viruses but would be far away from the transgene cassette in the case of the 1764 27- P2- 4- *vhs*- pR20.5 virus. However, until the *in vivo* expression profile of the 1764 27- P2- 4- *vhs*- pR20.5 virus has been definitively characterised these proposals remain speculative.



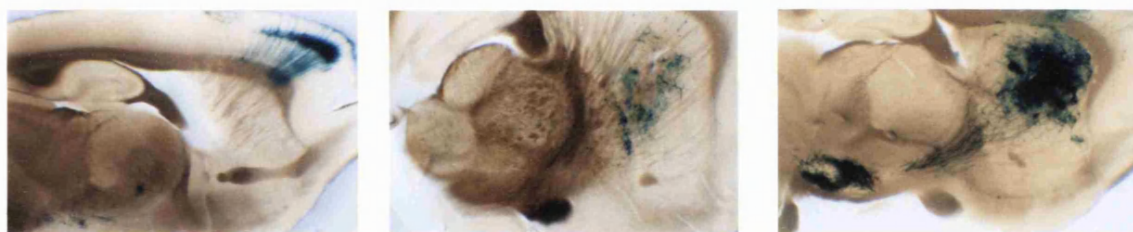
**Figure 7.6.1 Transgene expression from viruses containing the pR19*lacZ* cassette in the rat CNS *in vivo***

$2.5 \times 10^5$  pfu of either 1764 27- 4- pR19*lacZ*, 1764 27- pR19*lacZ* or 17+ 27- pR19*lacZ* were stereotactically injected into the rat striatum as described in section 2.6.3.1. Animals were perfusion fixed and the brains were sectioned in the parasagittal plane. Sections were 200 $\mu$ m thick. A) *lacZ* expression at 3 days post injection. B) *lacZ* expression at 1 week post injection. C) *lacZ* expression at 1 month post injection. The panel on the left shows transgene expression following injection of the 17+ 27- pR19*lacZ* virus, the middle panel the 1764 27- pR19*lacZ* virus and the panel on the right the 1764 27- 4- pR19*lacZ* virus, as indicated at the bottom of the figure.

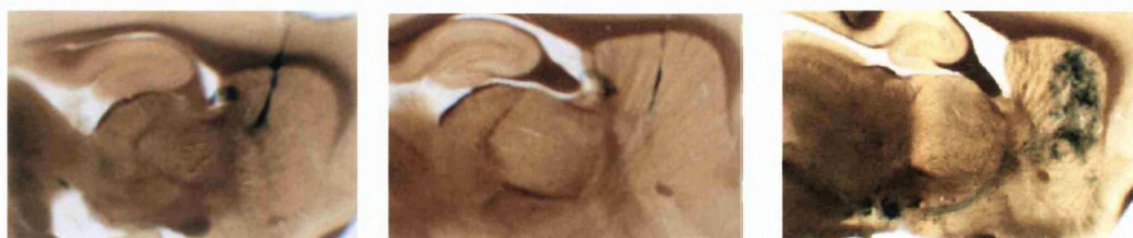
**A) 3 days post injection**



**B) 1 week post injection**



**C) 1 month post injection**



**17+27- pR19lacZ**

**1764 27- pR19lacZ**

**1764 27- 4- pR19 lacZ**

## 7.7 Discussion

This chapter has described the infection of primary neuronal cultures and the rat CNS *in vivo* with the HSV1 vectors constructed in this thesis. The non-toxic nature of the most disabled of these vectors was confirmed by the observation that it is non-toxic to neurons in culture, even at high MOI (figure 7.2.1) and at long times post infection (figure 7.5.1), at least as far as effects on neuronal morphology are concerned. A number of primary neuronal cultures were infected with the most disabled viruses. Surprisingly, it was found that certain types of culture appeared to be less susceptible to infection with the 1764 27- P2- 4- *vhs*- pR20.5 virus than others and that time in culture was an important parameter in allowing efficient transduction. However, infection with the 1764 27- 4-pR19GFP virus led to slightly higher levels of transduction efficiency than infection with the 1764 27- P2- 4- *vhs*- pR20.5 virus. Furthermore, infection with less disabled viruses containing either transgene cassette (17+27- and 1764 based viruses) consistently leads to almost 100% infectivity (Smith *et al.*, 1998b; Wagstaff *et al.*, 1999).

The tropisms of HSV1 were discussed in section 7.1. It is interesting to note that the neuronal culture systems which could be most reproducibly infected to high efficiencies were those derived from DRG neurons or hippocampal neurons, the natural site of HSV1 latency and most common site of experimental encephalitis respectively (Roizman and Sears, 1996; Damasio and Van Hoesen, 1985). It would therefore be attractive to hypothesise that the reason for the lower infectabilities of the other types of neuronal cultures might be that these cells did not have the same levels of the appropriate receptors to allow efficient HSV1 entry. Consistent with this hypothesis is the observation that neurons become more susceptible to infection after longer periods in culture, this perhaps allowing time for such receptors to be expressed. However, this suggestion is not consistent with the observation that the less disabled viruses 17+27- and 1764 are able to infect all types of neuronal cultures with high efficiency (Smith *et al.*, 1998b; Wagstaff *et al.*, 1999).

Furthermore, time of infection post plating is not important in the case of these less disabled viruses. It is perhaps therefore more likely that a restriction of gene

expression is occurring in some cell types but not others in the case of the most disabled virus. It is therefore possible that the infection efficiencies of some of the neuronal cultures used in this chapter are higher than reported in table XIII, i.e. only a small proportion of those neurons which are infected actually express the transgene. This restriction of gene expression could be attributable to the lack of transactivators in the most disabled virus and would therefore not apply to viruses 17+27- and 1764, both of which express high levels of the major HSV1 transactivators, ICP4 and ICP0. Such a hypothesis would be consistent with previously published observations which were discussed in chapter 6 (Samaniego *et al.*, 1998; Preston and Nicholl, 1997). Briefly, these authors separately reported a lack of CMV promoter driven transgene expression from viruses which did not express any IE gene products. However, if this were the explanation for the results presented in table XIII, it might be expected that no cells would express the transgene, rather than a low percentage expressing as was actually observed. Interestingly, the report by Samaniego and colleagues reported that GFP expression in infected non-neuronal tissue culture cells was not uniform, the majority of infected cells did not show any detectable CMV driven GFP expression but a small proportion showed abundant expression. The authors proposed that the CMV promoter might be active in the absence of viral transactivators only in cells which are at a particular stage of the cell cycle. As support for their suggestion, Samaniego and colleagues cited the observation that the intracellular environment which exists during certain stages of the cell cycle can reduce the requirement of HSV1 for ICP0 (Cai and Schaffer, 1991). Although this cell cycle theory cannot account for the observed expression of GFP in neurons reported in this chapter, it is interesting to speculate that a common mechanism might explain both these and the previously published findings.

Both the paper by Samaniego and colleagues and the paper by Preston and Nicholl observed that inhibition of protein synthesis by treatment of the cells with cycloheximide prior to infection was able to raise the level of transgene expression. This led both sets of authors to conclude that the lack of transgene expression was due to an active repression of the CMV promoter, possibly by a cellular protein which is only expressed in the absence of the viral transactivators. Based on this suggestion, it might be possible that the differences in expression levels between the various types of

neurons listed in table XIII might represent a cell type dependent difference in expression of this unidentified cellular protein.

A working hypothesis for all the observations described could therefore be that in the absence of viral transactivators, a cellular protein which is more abundant in cortical than DRG or hippocampal neurons actively represses the CMV promoter. To be fully consistent with all the observations reported, the levels of this cellular protein would have to be both developmentally regulated and also expressed at reduced levels after the neurons had been in culture for a week or more.

An experiment which could be conducted to investigate the reason for the lower level of GFP expression observed in certain neuronal cell types would be to infect the cultures as described and then superinfect them with a less disabled virus which did not express GFP. If it was found that transactivators from this superinfecting virus could cause GFP expression from the initially infected fully disabled virus, it could be concluded that the fully disabled virus had infected the neurons at high efficiency but that the CMV promoter had been repressed.

Figure 7.6.1 showed the expression of *lacZ* in the CNS of rats following injection of the disabled virus 1764 27- 4- pR19*lacZ*. The less disabled viruses 17+27- pR19*lacZ* and 1764 27- pR19*lacZ* were also injected as controls. Expression of *lacZ* was seen not only at the injection sites but at connected sites within the nervous system. At early times post injection, this was true for all three viruses although the patterns of transgene expression were very different. This point is discussed further below. Interestingly, only the most disabled virus 1764 27- 4- pR19*lacZ* continued to show transgene expression at both the site of injection and the substantia nigra at all time points tested. This indicates that the reason for the lack of continued expression in the viruses 17+27- pR19*lacZ* and 1764 27- pR19*lacZ* was probably not due to transcriptional shut off of the CMV promoter but for other reasons resulting from the toxicity of these less disabled viruses.

The most likely explanation for the observation of transgene expression in brain regions removed from the initial injection site is that the virus is retrogradely transported to the cell bodies of neurons innervating the striatum. Although very unlikely, it is a formal possibility that the transgene DNA or protein is being transported

rather than the virus. The fact that this is a theoretical possibility is demonstrated by a paper which reported delivery of *lacZ* expressing plasmid vectors from the periphery to spinal motor neurons (Sahenk *et al.*, 1993). However, this hypothesis would not easily be able to explain the different patterns of expression resulting from injection of the differently disabled viruses (as protein or DNA transport systems would be unlikely to be selective). It would also not be able to explain the fact that other vector systems such as adenovirus or AAV do not give such widespread gene expression.

The ability of wild-type HSV1 to undergo retrograde axonal transport has long been known (Bak *et al.*, 1977) and this property has been exploited by the use of wild type HSV1 as a tracer to assist in the delineation of neuroanatomical pathways (reviewed in Kuypers and Ugolini, 1990). Replication of the virus occurs after transneuronal transfer providing an amplification of the signal. Whilst this might be beneficial in tracing studies, the replicating virus is usually fatal to the experimental animal after only a short time scale.

The retrograde transport capabilities of replication compromised HSV1 vectors have also been described (Chiocca *et al.*, 1990; Huang *et al.*, 1992; Wood *et al.*, 1994; Maidment *et al.*, 1996; Jin *et al.*, 1996). Different patterns of staining have been reported to result from striatal injection of variably disabled HSV1 vectors expressing *lacZ*. When wild type HSV1 was injected into the striatum and the brain examined three days later, it was found that the virus had been retrogradely transported to neurons of the substantia nigra and cortex and very little virus could be detected at the site of injection (Bak *et al.*, 1977). Striatal injection with a *tk* deleted virus (replication competent only in dividing cells) was found to transduce a few cells at the injection site but with no spread to other sites in the brain. Likewise, injection with an ICP4 deficient virus (replication incompetent) showed some staining along the needle track but no expression in more distant cells or connected sites within the nervous system. In both of these cases, expression was completely undetectable by 14 days post injection. However, striatal injection of an ICP0 deficient virus (replication compromised only) demonstrated spread away from the site of delivery, predominantly giving gene expression the dendrites and axons of pyramidal neurons of the cortex. A later study by the same group using the same ICP0 deficient virus reported a small number of

transduced cells in the substantia nigra following striatal injection (Huang *et al.*, 1992). However, it was reported that these transduced cells were predominantly non-neuronal. While these differences in transgene expression pattern are obviously due to some initial replication of the ICP0 deficient but not the *tk* or ICP4 deficient viruses, it is interesting to note the cortical expression pattern of the minimally disabled, ICP0 deficient toxic virus. Chiocca *et al.* proposed that this pattern of labelling was due to retrograde transport of the virus from the striatum or sites along the injection track such as the corpus callosum.

In apparent contrast to the report by Chiocca and colleagues, an ICP4 deleted *lacZ* expressing virus injected into the striatum was reported to cause no staining at the injection site but to transduce cells in the substantia nigra (Maidment *et al.*, 1996). However, an examination of the photographs in this paper reveals that this nigral staining is very minimal with only a few transduced cells. This level of transgene expression could, for example, have been missed or not thought to be significant in the report by Chiocca. Another possible explanation for this apparent discrepancy could be that the two reports used different promoters to drive the expression of *lacZ* from the ICP4 deficient virus (the ICP6 promoter in the paper by Chiocca *et al.* and the MMTV LTR in the paper by Maidment *et al.*).

Several HSV1 amplicon systems have also been found to lead to retrograde transport from the site of injection to connected sites within the nervous system. The first of these to be published described injections into the striatum, dentate gyrus or cerebellar cortex and reported resultant labelling of the injection site in each case (Wood *et al.*, 1994). The authors also reported substantial labelling of the substantia nigra following striatal injection, the supramammillary nucleus following injection into the dentate gyrus and the locus coeruleus following injection into the cerebellar cortex. Other brain areas were also labelled, but in a less efficient manner. However, significant tissue necrosis was apparent by 5 days post injection at all the primary injection sites described. A more recent analysis of the spatial pattern of *lacZ* expression by an HSV1 amplicon found that the choice of promoter was a crucial determinant of whether significant expression from retrogradely transduced sites was observed (Jin *et al.*, 1996). When the ICP22/47 promoter was used to drive transgene expression, the

injection site (striatum or substantia nigra) was labelled at short time points after injection but limited retrograde transport was seen. However, when the TH promoter was used to drive *lacZ* expression and the vector was injected into the striatum, more substantial and longer lasting staining of both the injection site and the substantia nigra was observed. This reported expression pattern from the TH promoter in an amplicon vector has been confirmed several times since (Song *et al.*, 1997; Song *et al.*, 1998; Wang *et al.*, 1999).

To summarise the foregoing discussion, it appears that wild type virus injected into the striatum is not detected at the site of injection but is transported to the substantia nigra and cortex (Bak *et al.*, 1977). Replication attenuated, ICP0 deficient virus (Chiocca *et al.*, 1990) injected into the striatum is not transported to the substantia nigra but labels the site of injection and is transported to neurons in the cortex. In contrast, an ICP4 deficient virus injected into the striatum does not label the site of injection but in some circumstances is transported to the substantia nigra with low efficiency (Chiocca *et al.*, 1990; Maidment *et al.*, 1996). An amplicon vector injected into the striatum caused significant toxicity but showed high level staining of the injection site and of the substantia nigra (Wood *et al.*, 1994; Jin *et al.*, 1996) and occasionally of neurons in the cortex (Wood *et al.*, 1994). Although not addressed in the paper by Wood and colleagues, it could be speculated that the cortical labelling might be due to contamination of the stocks with helper virus. Consistent with this suggestion, this cortical staining was only detected following the highest titre injections and only in a small proportion of the animals.

If the data presented in figure 7.6.1 of this chapter are considered in light of the conclusions from the preceding paragraph, it is interesting to note that the staining resulting from injection of the virus 17+27-pR19*lacZ* is more consistent with the expression pattern of a replication attenuated rather than a replication incompetent virus. This might suggest that this cortical staining pattern is associated with high toxicity rather than replication competence. The striatum, substantia nigra and cortex are all stained at 3 days post injection. However, by 1 week post injection, the striatum is not stained and it appears that all the virus has been retrogradely transported, the majority to neurons in the cortex although some staining of the substantia nigra can still



be seen. By 1 month post injection only low level labelling of a few cells is seen. However, in the case of the intermediately disabled virus, 1764 27-pR19*lacZ*, a completely different pattern of expression is observed. Here there is intense staining of the striatum and some staining of the substantia nigra at 3 days post injection. This pattern is maintained but significantly reduced by 1 week post injection and again only low level labelling is seen at 1 month post injection. Notably, no cortical neurons other than those in the needle track were stained following striatal injection with this virus at any time point. Since ICP4 is the major transactivator of HSV1 and its deletion is known to prevent the expression of the vast majority of the viral genome, it would be expected that a virus deleted for ICP4 only would express considerably less HSV1 gene products than one deleted for ICP27 only. It could therefore be proposed that the ICP4 deficient viruses described in the reports by Chiocca and Maidment are intermediate in their toxicity (and therefore their expression pattern) between the 17+27-pR19*lacZ* and the 1764 27- pR19*lacZ* viruses described in this thesis.

The 1764 27- 4- pR19*lacZ* virus is considerably less toxic than any of the viruses so far discussed and as such the *lacZ* staining is much more intense in both the striatum and the substantia nigra and the expression is maintained for at least 1 month post injection. Consistent with the emerging consensus of expression patterns discussed above, very few cortical neurons other than those in the injection track were labelled with this virus.

In summary, this chapter has described the *in vitro* and *in vivo* capabilities of the viruses constructed in this thesis to deliver genes to neurons. The most disabled virus gives highly effective gene delivery *in vitro* and *in vivo*, not only to the injection site but to connected sites in the nervous system, presumably by retrograde transport. Less disabled viruses, whilst giving effective transduction *in vitro*, gave less widespread expression *in vivo* and this was considerably less efficient in the long term. The multiply disabled viruses constructed in this thesis therefore represent an effective and non-toxic transduction system for neurons both *in vitro* and *in vivo*.

## **CHAPTER 8**

# **CONSTRUCTION AND PRELIMINARY CHARACTERISATION OF VIRUSES CONTAINING GENES RELEVANT TO ALZHEIMER'S DISEASE**

## 8.1 Introduction

AD is an incompletely understood CNS disorder characterised by the presence of neuritic A $\beta$  plaques and neurofibrillary tangles of hyperphosphorylated tau. As discussed in the general introduction, five genes are now known to cause or modulate AD. These are APP, PS1, PS2, ApoE and  $\alpha$ 2M. Specific mutations in any of the first three genes cause AD with 100% penetrance whereas polymorphisms in the latter two are age-dependent risk factors.

A number of transgenic mouse models have been generated in an attempt to reproduce the biochemical and behavioural abnormalities characteristic of AD. The main features of several of these models were discussed in section 1.3. However, whilst these mice show extensive amyloid deposition, none of them exhibit the overt neuronal loss or neurofibrillary tau tangles which are pathological hallmarks of the human disease. This has led to the criticism that the currently available transgenic mice are models of amyloid deposition rather than AD *per se*. Possible reasons for the lack of tau pathology in these mice include the fact that there are substantial differences between the isoforms of tau found in human and rodent brains and that rodents have a considerably shorter lifespan than humans. It is therefore possible that human but not rodent tau is prone to hyperphosphorylation and tangle formation in response to unknown pathological stimuli associated with AD. This hypothesis was discussed in detail in section 1.3.5. It is also possible that while PS and APP mutations lead to elevated A $\beta$  deposition in both humans and mice, such A $\beta$  deposition is not the primary cause of AD, but merely a by-product of other as yet unidentified pathological molecular mechanisms.

The viral vectors described in this thesis offer the potential to explore gene interactions relevant to AD in non-rodent models. It would represent a significant advance if it could be shown that mutations in APP and/or PS1 were sufficient to cause full amyloid and neurofibrillary pathology in, for example, the primate CNS. This model could then be used to define the pathways which ultimately lead to the pathology of AD. Alternatively, the vectors described could be used for numerous *in vitro* gene delivery applications to rapidly evaluate the effects of various mutations in APP and/or PS1 on various aspects of normal neuronal functioning.

This chapter describes the construction and characterisation of a panel of disabled viruses expressing APP and/or PS1 wild type or mutants in various combinations. Preliminary *in vitro* experiments using rodent and non-rodent neurons are described and the potential for *in vivo* experiments with these viruses is discussed.

## **8.2 Cloning of APP and PS1 wild type or mutants into the pR20.5 cassette**

The aim of this section was to clone either APP WT or mutant in combination with a reporter gene, PS1 WT or mutant in combination with a reporter gene and the four possible combinations of APP WT or mutant in combination with PS1 WT or mutant into shuttle plasmids for recombination into the viral genome. Since the eventual aim was to recreate AD pathology, the most aggressive mutants of APP and PS1 were used in the cloning. The APP Swedish mutation (APP Swe) was described in section 1.2.1. This double mutation is known to cause AD pathology with a mean age of onset of 52 years. The APP Swe mutant has been successfully used in the generation of transgenic mice which display A $\beta$  deposition and some behavioural deficits (Hsiao *et al.*, 1996). Cosmids containing the cDNA for the 695 amino acid isoform of human APP with and without the Swedish mutation were provided by Karen Hsiao, University of Minnesota.

The mutations in PS1 are generally homogenous in the pathological phenotype they cause, with the mean age of onset for all the identified PS1 mutations being between 45 and 55 years of age. A particularly aggressive PS1 mutation (L392V) has been identified which was reported to cause AD pathology in a 28 year old (Campion *et al.*, 1995). However, this mutation is not well-characterised and so was not used in this thesis. The A246E mutation is a more representative PS1 mutation, causing AD with an age of onset of 50 to 55 years (Sherrington *et al.*, 1995). This mutation has also been well studied and incorporated into the germline of transgenic mice where it was found to cause a selective increase in the production of A $\beta$ 42 (Borchelt *et al.*, 1996). PS1 WT and PS1 A246E were provided by Jill Richardson, Neurosciences Unit, Glaxo-Wellcome.

Chapter 6 described the construction and characterisation of viruses disabled for ICP27, ICP34.5, VP16 and ICP4 and containing the expression cassette pR19 or viruses disabled for ICP27, ICP34.5, VP16, ICP4, LAT P2 and *vhs* and containing the

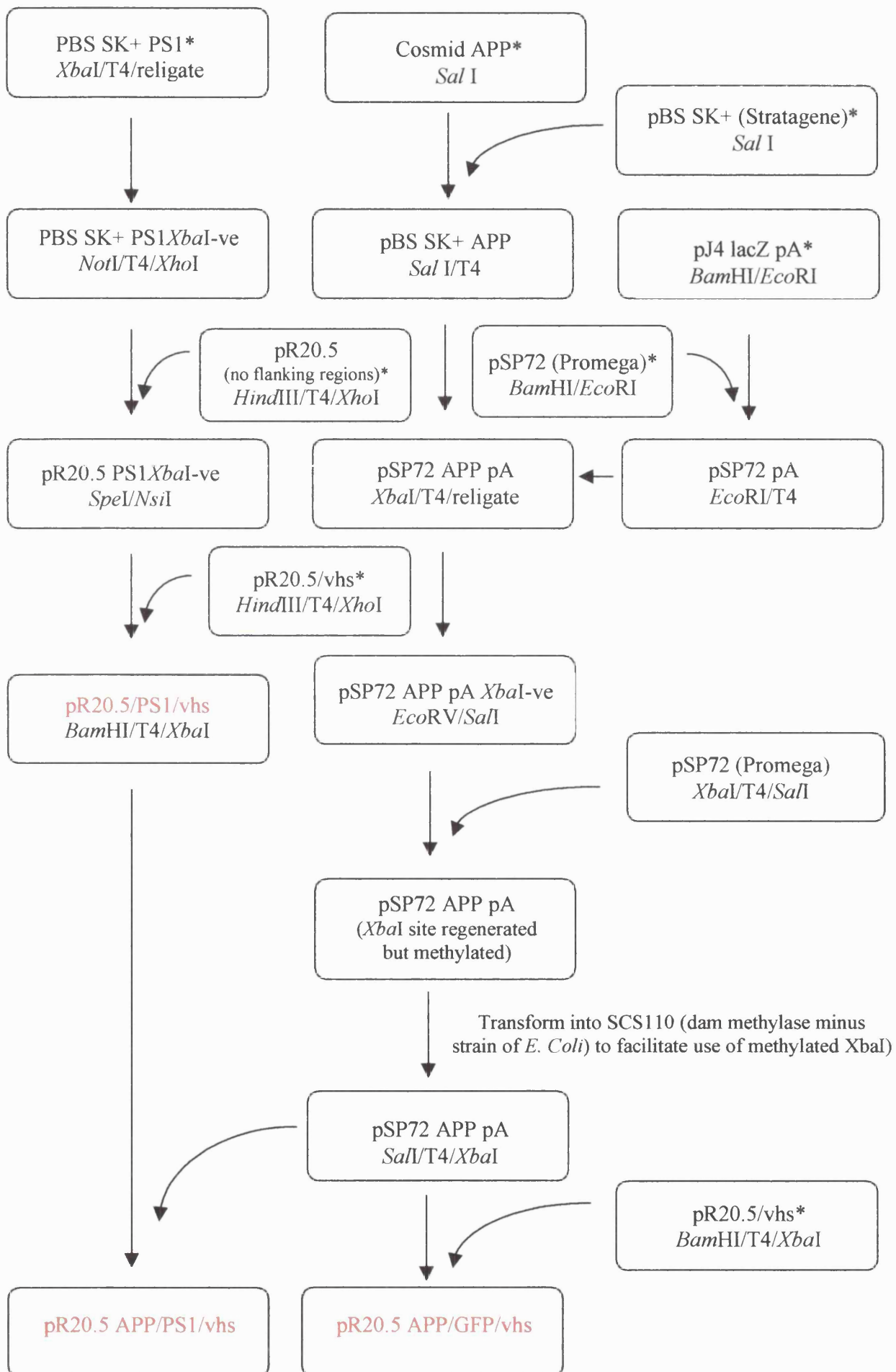
expression cassette pR20.5. Chapter 7 compared the expression profiles of these two reporter gene viruses over several months *in vivo*. A consideration of the data discussed in chapter 7 might suggest that pR20.5 containing viruses are less capable of reliably driving long term expression of transgenes *in vivo*. However, as the pR20.5 cassette but not the then available pR19 cassette had the capacity for the simultaneous expression of multiple exogenous genes and since the simultaneous expression of APP and PS1 was required in this project, these genes were cloned into the pR20.5 cassette.

APP WT, APP Swe, PS1 WT and PS1 A246E were all sequenced across the mutation site to confirm the presence of the mutation and then cloned into the pR20.5 shuttle plasmid either singly or in various combinations. The cDNAs for APP and PS1 are 2.5 and 1.5kb respectively and the APP sequence contains many of the commonly used restriction sites. This meant that the cloning of these genes into the pR20.5 cassette had to proceed by a lengthy and indirect route. The details of this cloning are shown in a flow diagram in figure 8.2.1. Briefly, APP was cloned out of the cosmid into the plasmid pBluescript in order to facilitate easier manipulation. The polyadenylation signal from pJ4*lacZ*pA was excised and cloned into pSP72 (Promega). pJ4*lacZ* is a plasmid which previously existed in the laboratory (constructed by insertion of the *lacZ* gene and its polyadenylation signal from pCH110 (Pharmacia) as a *Bam*HI/*Hind*III fragment into the *Kpn*I site of pJ4 (Morgenstern and Land, 1990). APP was then cloned into pSP72 upstream of this polyadenylation signal and then repositioned in pSP72 such that it could be excised with the polyadenylation signal for insertion into the pR20.5 cassette in *vhs* flanking regions in place of *lacZ*. Acquiring the polyadenylation signal from pJ4*lacZ*pA was necessary because an *Eco*RI site in the RSV promoter prevented the use of the same site at the end of *lacZ* in pR20.5/*vhs*. The latter stages of this part of the cloning were carried out in a dam methylase minus strain of *E.coli* to facilitate the later use of a regenerated but methylated *Xba*I site. This part of the cloning was repeated to generate shuttle plasmids pR20.5 APP WT/GFP/*vhs* and pR20.5 APP Swe/GFP/*vhs*. The PS1 half of the cloning was achieved by first destroying an *Xba*I site in pBS and then inserting PS1 into the pR20.5 cassette without any flanking regions. The *Xba*I site was destroyed so that another *Xba*I site which would be introduced later in the cloning would be unique. PS1 was not directly inserted into pR20.5 in *vhs*

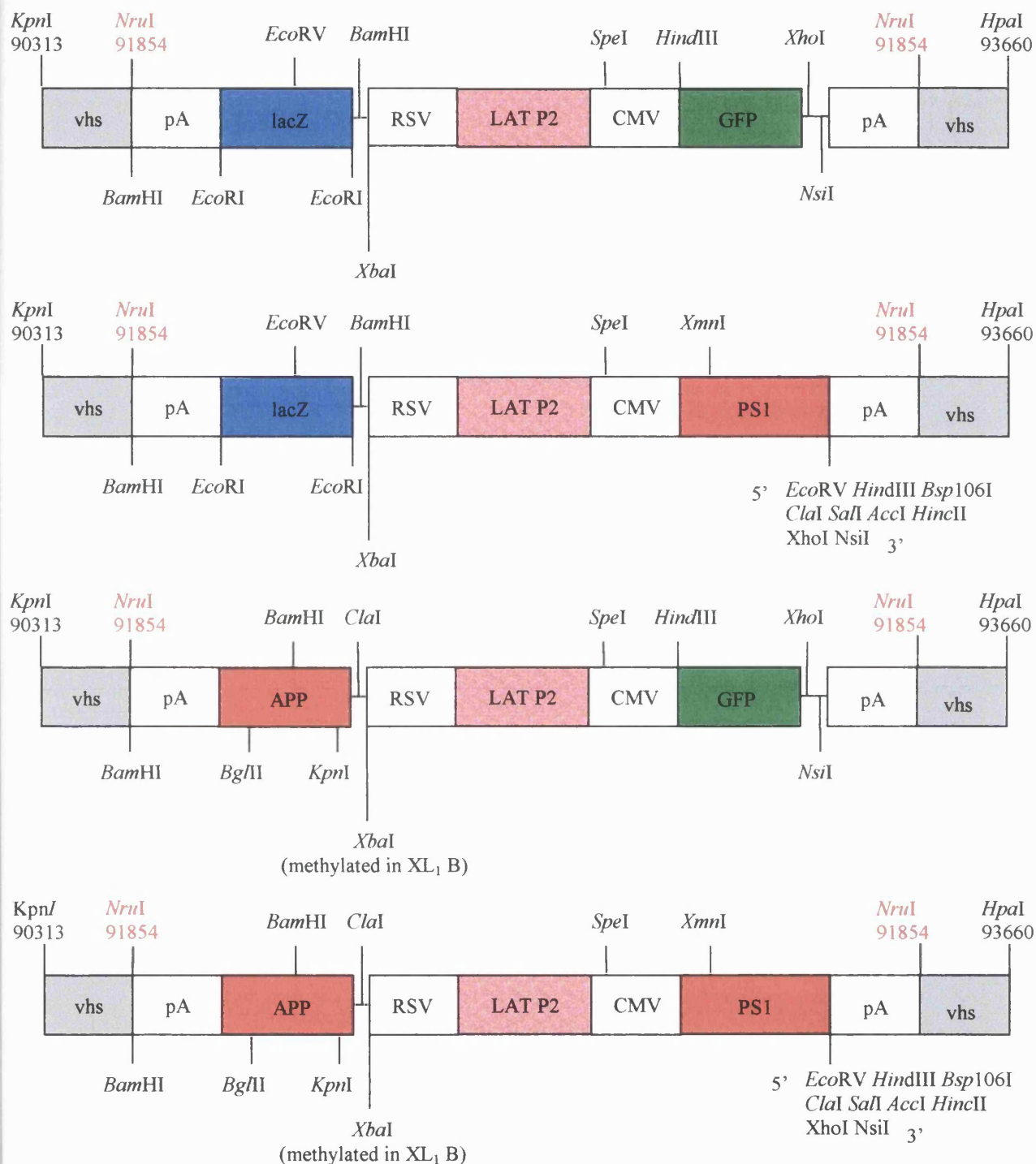
flanking regions as there were no sites at the 5' end of PS1 in pBluescript which did not cut in the *vhs* flanking regions. Instead, a unique *SpeI* site in the CMV promoter and a unique *NsiI* site at the 3' end of PS1 were used to transfer this half of the 20.5 cassette (now containing PS1) to the equivalent construct in *vhs* flanking regions. This part of the cloning was also repeated to generate shuttle plasmids pR20.5/PS1 WT/*lacZ/vhs* and pR20.5/PS1 A246E/*lacZ/vhs*. The now unique *XbaI* site in the PS1 half of the cloning and the regenerated (now unmethylated) *XbaI* site in the APP half of the cloning were now used to put all the different constructs together in various combinations to generate a set of eight shuttle plasmids. A map for one of each type of shuttle plasmid is shown in figure 8.2.2.

**Figure 8.2.1 Cloning of APP and PS1 into the shuttle cassette pR20.5/*vhs***

The steps involved in the cloning are summarised in a flow diagram. The final plasmids (APP or PS1 singly or APP and PS1 together) are shown in red. Intermediates in the cloning are shown in black. Asterisks indicate plasmids which were previously existing in the laboratory or have been described elsewhere in this thesis. Only one APP and PS1 isoform are indicated in the diagram but this cloning was repeated several times in order to generate the complete set of eight shuttle plasmids as shown in figure 8.3.1.







**Figure 8.2.2 Maps of shuttle plasmids containing APP and PS1**

A map of pR20.5 in *vhs* flanking regions is shown with sites used in the cloning indicated. A map of one of each type of shuttle vector containing APP and/or PS1 is shown with selected restriction sites indicated. The complete set of APP/PS1 shuttle plasmids is shown in figure 8.3.1. The HSV1 *NruI* site indicated in red was destroyed in the cloning and is shown for orientation purposes only.

### 8.3 Recombination of APP/PS1 shuttle plasmids into the 1764 27- or 1764 27- P2- 4- viral backbones

As was mentioned in section 6.2, the single and then the double shuttle plasmids were initially recombined into the *vhs* locus of the less disabled virus 1764 27- and the viruses plaque purified. Although the pR20.5 cassette appeared to be relatively stable in the *vhs* locus (a site far away from the LAT region), the lack of any reporter genes in the pR20.5 APP/PS1 double viruses means that aberrant recombination events could easily go unnoticed. In order to ensure that any results with these viruses were meaningful, the viruses would have to be continually screened for the presence of the inserts. As a positive signal on a western blot does not indicate that the population is 100% correct, but only that it is not 100% incorrect, the detailed structures of the viral genomes would have to be confirmed by Southern blots at regular intervals. This was clearly an undesirable situation.

Whilst the cloning of the shuttle plasmids described above and the purification of these less disabled viruses was being carried out, the work described in chapters 5, 6 and 7 had continued in parallel, indicating that ICP4 should also optimally be deleted from the backbone virus. The cell line described in chapter 4 had also been constructed, meaning that ICP4 deleted viruses could now be easily purified and grown to high titres in culture. It was therefore decided to discontinue work on the 1764 27- APP/PS1 viruses and remake them in the backbone of the virus described in chapter 5, 1764 27- P2- 4- pLU/4. This would not only offer significant advantages in terms of lack of toxicity to target cells, but the lack of endogenous LAT P2 regions in the viral backbone removes the possibility for aberrant homologous recombination events.

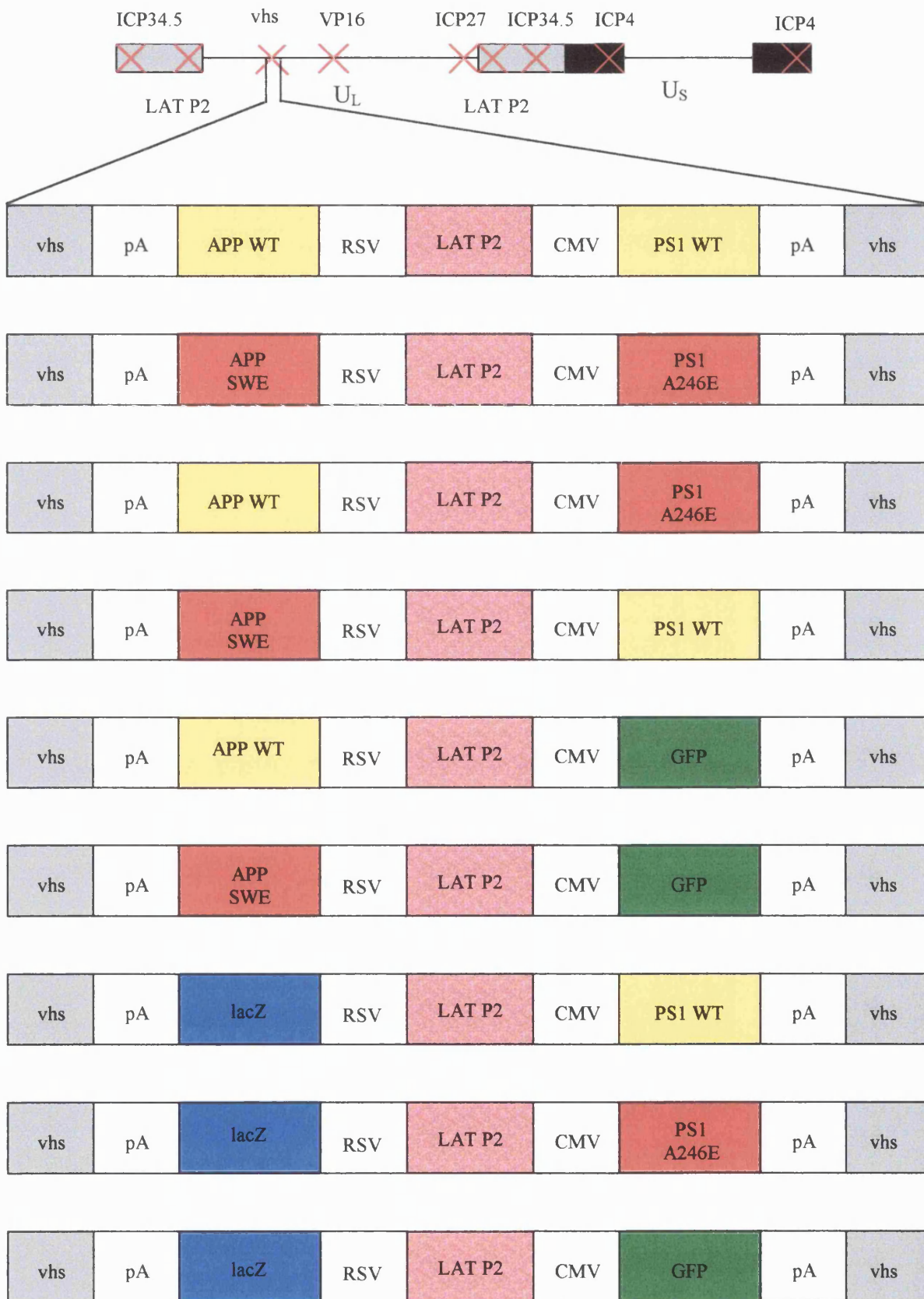
The single APP or PS1 plus reporter gene plasmids (pR20.5/PS1WT/*lacZ*/*vhs*, pR20.5/PS1A246E/*lacZ*/*vhs*, pR20.5/APPWT/GFP/*vhs* and pR20.5/APPSwe/GFP/*vhs*) were linearised using a unique *SspI* site in the plasmid backbone and cotransfected with infectious 1764 27- P2- 4- pLU/4 viral DNA. Recombinant plaques were identified by their ability to express GFP (for APP WT or APP Swe plasmids) or *lacZ* (for PS1 WT or PS1 A246E plasmids) and plaque purified. Maps of the resulting viruses, 1764 27- P2- 4- *vhs*- pR20.5 APPWT/GFP, 1764 27- P2- 4- *vhs*- pR20.5 APPSwe/GFP, 1764 27-

P2- 4- *vhs*- pR20.5 PS1WT/*lacZ* and 1764 27- P2- 4- *vhs*- pR20.5 PS1A246E/*lacZ* are shown in figure 8.3.1.

The single APP/GFP viruses were then used as a starting point for the generation of the double APP/PS1 viruses. Infectious “green” viral DNA was prepared from 1764 27- P2- 4- *vhs*- pR20.5 APP WT/GFP and 1764 27- P2- 4- *vhs*- pR20.5 APP Swe/GFP. The double APP/PS1 plasmids were linearised using a unique *SspI* site in the plasmid backbone and co-transfected with the appropriate viral DNA (i.e. any APP/PS1 plasmid containing APP Swe was cotransfected with the viral DNA containing APP Swe and any APP/PS1 plasmid containing APP WT was cotransfected with the viral DNA containing APP WT). Recombinant plaques containing both APP and PS1 were identified as white plaques and purified away from background of green plaques. Maps of the resulting viruses, 1764 27- P2- 4- *vhs*- pR20.5 APP WT/PS1 WT, 1764 27- P2- 4- *vhs*- pR20.5 APP Swe/PS1 WT, 1764 27- P2- 4- *vhs*- pR20.5 APP WT/PS1 A246E and 1764 27- P2- 4- *vhs*- pR20.5 APP Swe/PS1 A246E are shown in figure 8.3.1.

**Figure 8.3.1 Complete set of 1764 27- P2- 4- *vhs*- APP/PS1 viruses**

Maps of all the 1764 27- P2- 4- *vhs*- APP/PS1 viruses are shown. The negative control virus 1764 27- P2- 4- *vhs*- pR20.5 is shown for comparative purposes. WT genes are shown in yellow and mutants in red. All the insertions were made into the UL41, the gene encoding *vhs*. Equivalent insertions were made into the backbone 1764 27- generating eight identical viruses but without ICP4 or the endogenous LAT P2 deleted. These viruses are not shown in the figure and were not used for any experiments.



#### **8.4 Viruses express APP and PS1**

Western blots were carried out in order to confirm that the eight viruses described in the previous section all expressed APP and/or PS1. Being a multiple transmembrane protein, PS1 is prone to aggregation. A novel extraction method was developed in order to enable reliable and consistent PS1 western blots to be carried out<sup>2</sup>. This extraction method is based on suggestions from Paul Mathews, Nathan Kline Institute, New York (personal communication) and is detailed in section 2.5.2. Briefly, protein samples were extracted in buffer containing 2M urea by shearing through a hypodermic needle. Samples were not boiled but warmed to 37°C before electrophoresis on a SDS-PAGE gel also containing 2M urea. Figures 8.4.1 and 8.4.2 show western blots for APP and PS1 expression from the double and single APP/PS1 expressing viruses respectively.

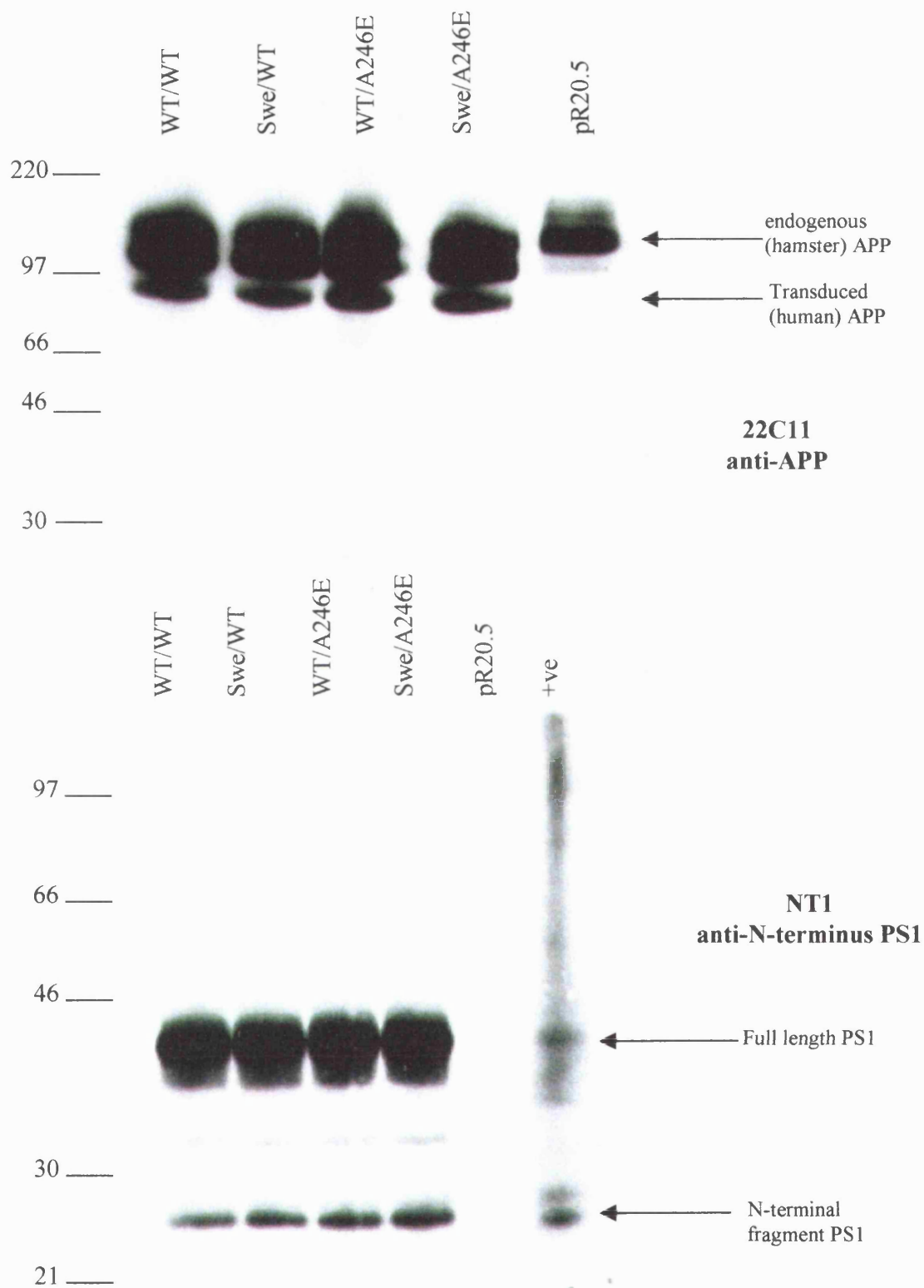
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<sup>2</sup> This extraction method is also useful for extracting other “fragile” proteins, for example HSV1 ICP4.

**Figure 8.4.1 APP and PS1 expression from the 1764 27- P2- 4- *vhs*- double APP/PS1 expressing viruses**

27/12/M:4 cells were infected at an MOI of 0.01 and harvested 48 hours post infection. Samples were prepared for western blot analysis and separated by SDS-PAGE as described in sections 2.5.1 and 2.5.3 (for APP) or 2.5.2 and 2.5.3 (for PS1). The anti-APP antibody 22C11 was purchased from Boehringer Mannheim and the anti-PS1 antibody NT1 and the PS1 positive control were a gift from Paul Mathews, Nathan Kline Institute, New York. NT1 is raised against an epitope in the N-terminus of PS1 and therefore also reacts with the 28kDa N-terminal fragment.

WT/WT	1764 27- P2- 4- <i>vhs</i> - APP WT/PS1 WT
Swe/WT	1764 27- P2- 4- <i>vhs</i> - APP Swe/PS1 WT
WT/A246E	1764 27- P2- 4- <i>vhs</i> - APP WT/PS1 A246E
Swe/A246E	1764 27- P2- 4- <i>vhs</i> - APP Swe/PS1 A246E
pR20.5	1764 27- P2- 4- <i>vhs</i> - pR20.5
+ve	extract from L cells stably overexpressing PS1 (PS1 blot only)





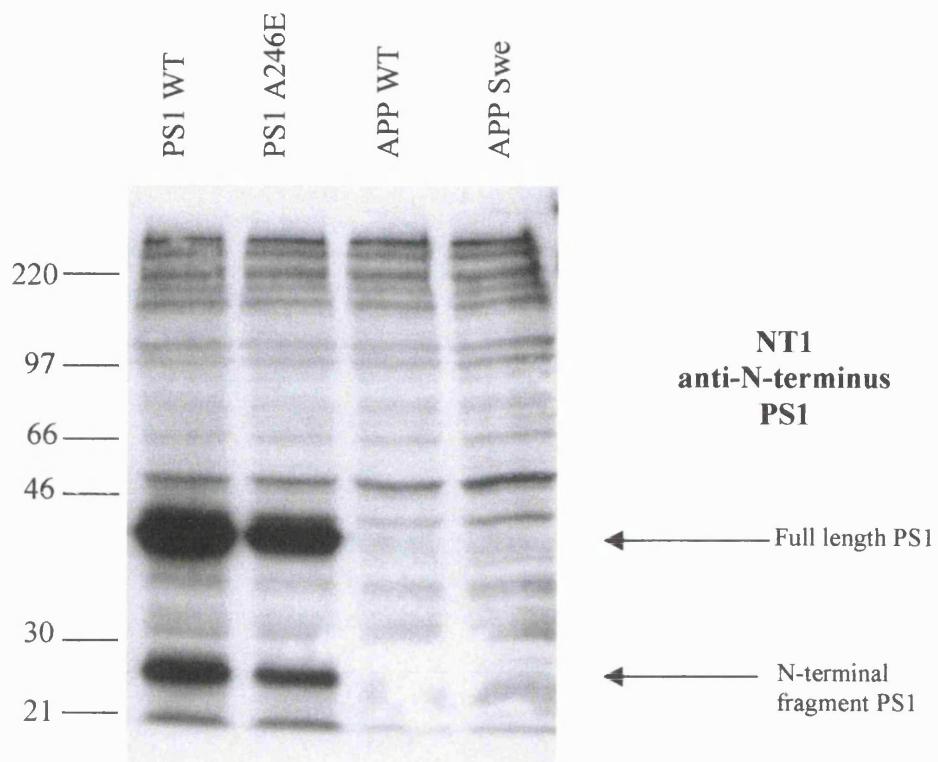
**Figure 8.4.2 APP and PS1 expression from the 1764 27- P2- 4- *vhs*- single APP or PS1 expressing viruses**

27/12/27:4 cells were infected at an MOI of 0.01 and harvested 48 hours post infection. Samples were prepared for western blot analysis and separated by SDS-PAGE as described in sections 2.5.1 and 2.5.3 (for APP) or 2.5.2 and 2.5.3 (for PS1). The anti-APP antibody 22C11 was purchased from Boehringer Mannheim and the anti-PS1 antibody NT1 was a gift from Paul Mathews, Nathan Kline Institute, New York. NT1 is raised against an epitope in the N-terminus of PS1 and therefore also reacts with the 28kDa N-terminal fragment.

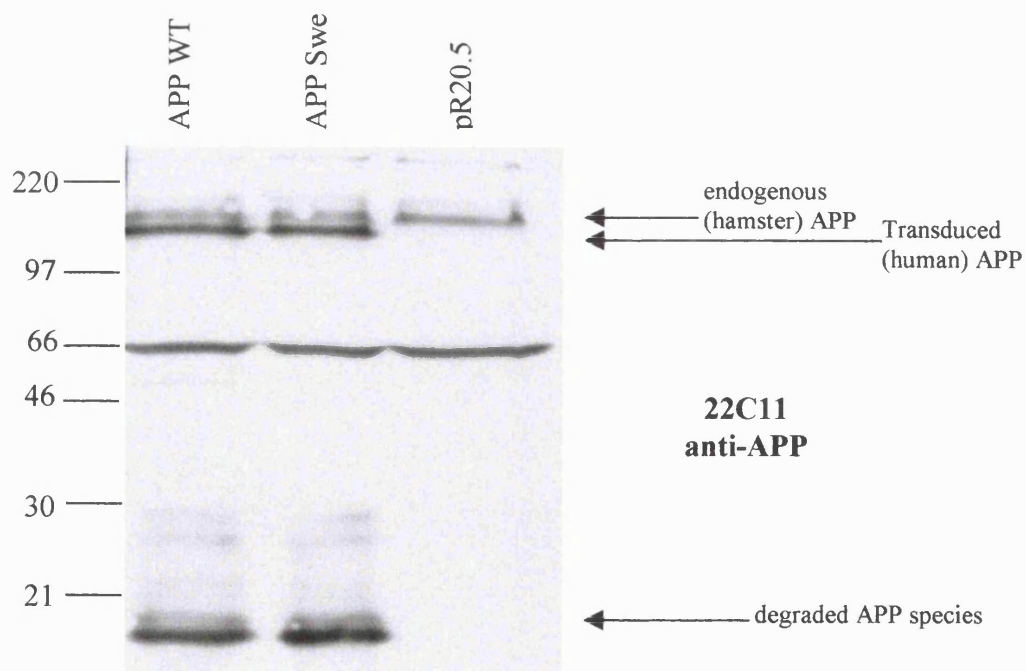
The difference in cell line between this figure and figure 8.5.1 (27/12/27:4 as opposed to 27/12/M:4) probably accounts for the differences in signal intensity between the two figures. In other experiments the two sets of viruses were found to express equally high levels of both transgenes.

A)	PS1 WT	1764 27- P2- 4- <i>vhs</i> - PS1WT/ <i>lacZ</i>
	PS1 A246E	1764 27- P2- 4- <i>vhs</i> - PS1A246E/ <i>lacZ</i>
	APP WT	1764 27- P2- 4- <i>vhs</i> - APP WT/GFP
	APP Swe	1764 27- P2- 4- <i>vhs</i> - APP Swe/GFP
B)	APP WT	1764 27- P2- 4- <i>vhs</i> - APP WT/GFP
	APP Swe	1764 27- P2- 4- <i>vhs</i> - APP Swe/GFP
	pR20.5	1764 27- P2- 4- <i>vhs</i> - pR20.5

**A)**



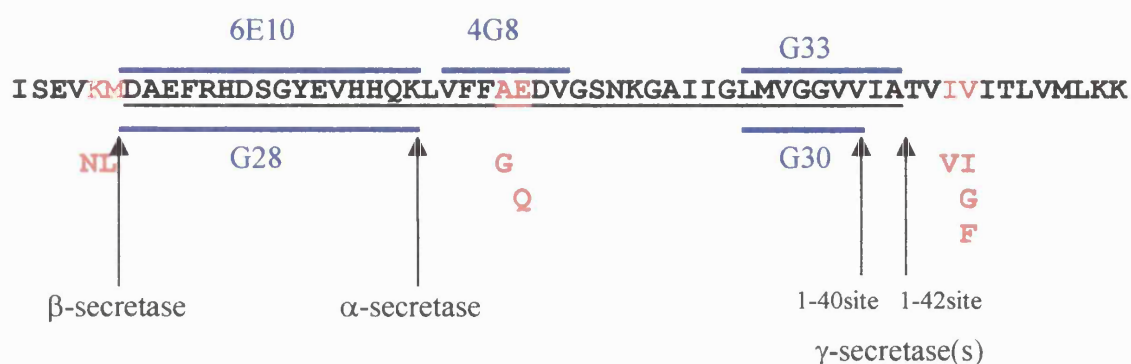
**B)**



## 8.5 A $\beta$ ELISAs on supernatants of cells infected with the APP/PS1 expressing viruses

A $\beta$  species are small secreted peptides (approximately 4kDa) which are usually quantified by ELISA or immunoprecipitation. In order to establish whether the human APP and PS1 being expressed by the disabled viruses were in a functionally active form, ELISAs were carried out to assay for A $\beta$  secretion. The sandwich ELISA employed was developed by the Neurosciences Unit, Glaxo-Wellcome. The regions of A $\beta$  recognised by the various antibodies used are indicated in figure 8.5.1.

Parental human embryonic kidney 293 cells (Graham *et al.*, 1977) or cells which had been stably transfected to constitutively express human APP WT or human APP Swe (previously constructed at Glaxo-Wellcome) were infected at an MOI of 1 with a selection of the 1764 27- P2- 4- *vhs*- double APP/PS1 viruses. 48 hours post infection, the supernatants were harvested and prepared for ELISA as described in section 2.5.8. ELISAs were carried out for total A $\beta$ , A $\beta$ 1-40 and A $\beta$ 1-42 using antibodies specific for each peptide as shown in figure 8.5.1. The standard curves for the ELISAs are shown in figure 8.5.2 and the results of the ELISAs for total A $\beta$  and A $\beta$ 1-40 on 293 APP Swe and 293 APP WT cells are shown in figure 8.5.3. The ELISA from the supernatants of parental 293 cells was completely negative for all A $\beta$  peptides so this data set is not included in figure 8.5.3.



**Table XIV: Antibody combinations used in the Aβ sandwich ELISA**

ELISA	Capture antibody	Secondary antibody	Tertiary antibody
Total	4G8	G28	Anti-rabbit-HRP
Aβ1-40	G30	6E10	Anti-mouse-HRP
Aβ1-42	G33	6E10	Anti-mouse-HRP

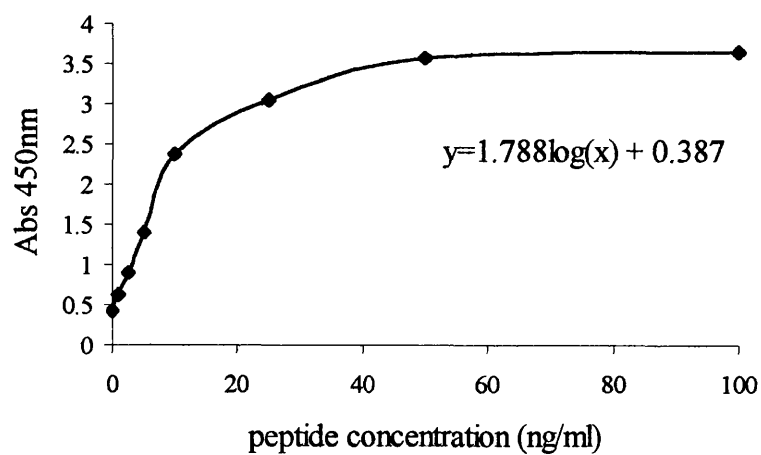
#### Figure 8.5.1 Antibodies used in the Aβ sandwich ELISA

The transmembrane region of APP is indicated with the Aβ sequence underlined in black. Amino acids shown in red indicate the mutations associated with AD or related dementias (see section 1.2.1). The epitopes against which the antibodies used in the ELISA were raised are indicated in blue. Antibodies with the prefix G were generated by Glaxo-Wellcome. 6E10 and 4G8 were purchased from Senetek plc, St. Louis, Missouri. Table XIII shows the various combinations of antibodies which were used in each of the sandwich ELISAs. Details of the ELISA protocol can be found in section 2.5.8.

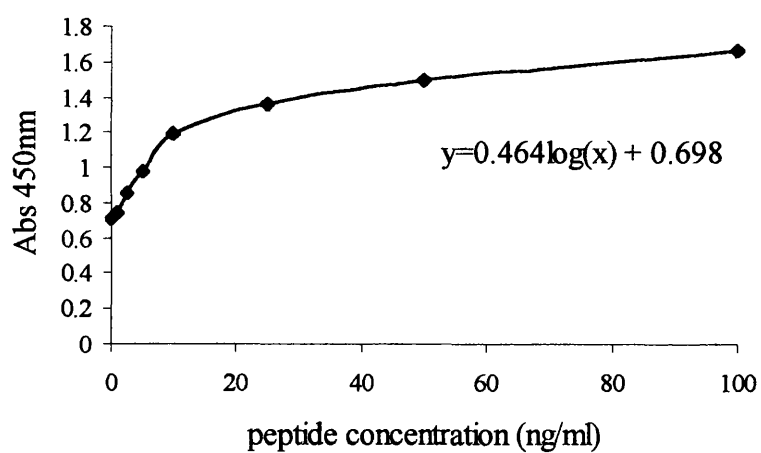
**Figure 8.5.2    Standard curves for the A $\beta$  ELISA**

Standard curves for the total A $\beta$ , A $\beta$ 1-40 and A $\beta$ 1-42 ELISAs are shown. Best fit curves and corresponding equations were fitted by AssayZap (Biosoft, Cambridge, U.K.).

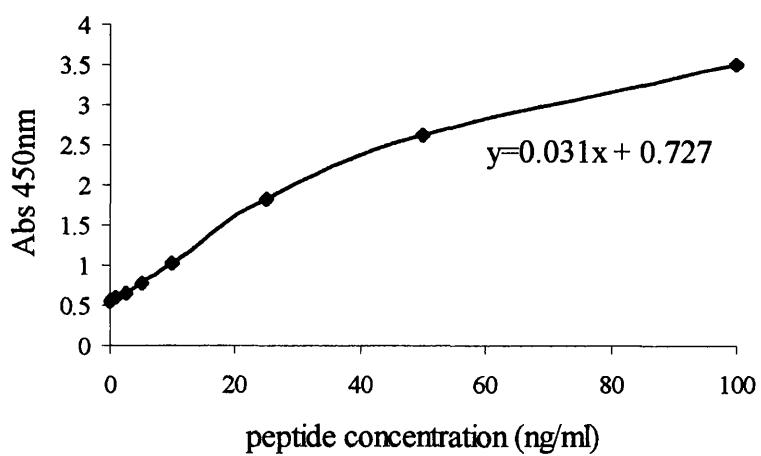
Standard curve for A $\beta$ 1-40 ELISA



Standard curve for A $\beta$ 1-42 ELISA



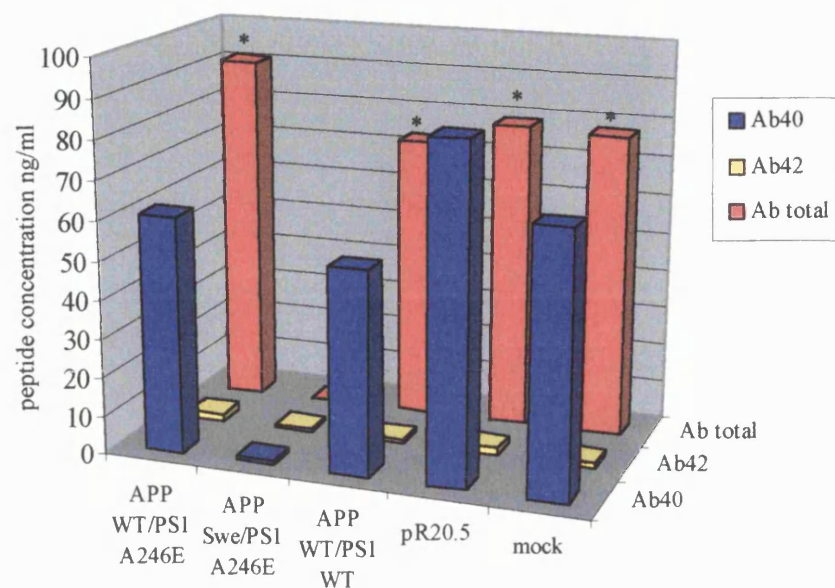
Standard curve for total A $\beta$  ELISA



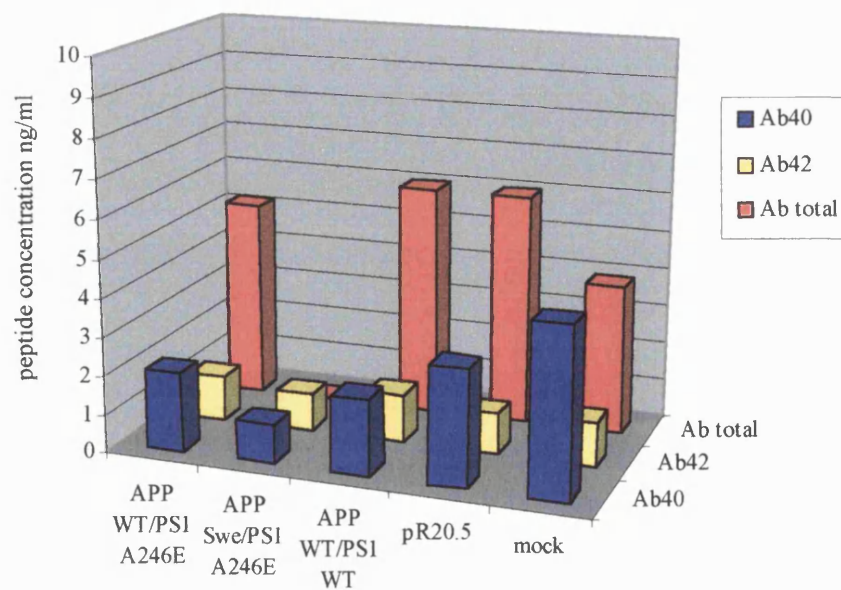
**Figure 8.5.3 ELISAs for A $\beta$ 1-40 or total A $\beta$  secreted from 293 APP Swe or 293 APP WT cells infected with the double APP/PS1 viruses**

The graphs show the amount of A $\beta$  in ng/ml secreted from 293 APP Swe or 293 APP WT cells 48 hours post infection at an MOI of 1 with the 1764 27- P2- 4- *vhs*- APP Swe/PS1 A246E, APP WT/PS1 WT, APP Swe/PS1 WT or pR20.5 viruses. Results are presented as amount of A $\beta$  in ng/well. Asterisks indicate that these 4 readings were taken from points past the linear range of the standard curve and are therefore beyond the sensitivity of this assay. It can also be seen that the apparent amount of A $\beta$ 1-40 or A $\beta$ 1-40 secreted is occasionally higher than the total A $\beta$  secreted. The A $\beta$ 1-42 results were not above background for any sample so it was assumed that this response was non-specific and reflected differences in sensitivity between the different assays.

### A) HEK 293 APP Swe cells



### B) HEK 293 APP WT cells





From the information discussed in chapter 1, it would be expected that viruses expressing APP Swe would cause an increase in total A $\beta$  and viruses expressing PS1 A246E would cause a specific increase in the ratio of A $\beta$ 1–42 to A $\beta$ 1–40. It can be seen from figure 8.5.3 that this was clearly not observed in this experiment. In contrast, the double mutant virus expressing APP Swe and PS1 A246E appeared to completely prevent A $\beta$  expression from the 293 cells, even when these cells are overexpressing APP Swe and therefore normally secreting high levels of A $\beta$ . This surprising observation was reproduced three times using two different stocks of the virus, both of which were known to express high levels of APP and PS1. It therefore appears that not only is the double mutant virus not causing an increased secretion of A $\beta$ , but somehow this virus is preventing  $\beta$ -secretase processing of the cellularly derived APP Swe. Two possible explanations will be considered below.

Firstly, it is possible that the very high level of expression of APP Swe during viral growth on 27/12/M:4 cells has led to the accumulation of degraded APP peptides containing the  $\beta$ -secretase target sequence. If these peptides were carried over to the 293 Swe cells at the time of infection, it is possible that they out-competed the cellular and virally derived APP Swe for a limited pool of  $\beta$ -secretase, resulting in a “dead-end” interaction with the enzyme. Potential support for this hypothesis comes from the observation that small peptides which react with anti-APP antibodies are seen on western blots following infection with the APP expressing viruses (for example see the APP western blot in figure 8.4.2). These small peptides are assumed to be partially degraded APP molecules resulting from the high level of expression from the viruses during growth on complementing cell lines. Furthermore, it is known that small peptides containing the  $\beta$ -secretase target sequence can be efficiently cleaved by the enzyme (Vassar *et al.*, 1999). However, this hypothesis is very unlikely to explain the results observed here as any degraded peptides which were carried over would be extracellular whereas the  $\beta$ -secretase would be intracellular.

A more likely explanation for the apparent inhibition of A $\beta$  secretion caused by the double mutant virus might be that infection with this virus “overloaded” the system

or the mutant APP and mutant PS1 mediated toxic effects which prevented A $\beta$  secretion.

A further potential contributing factor to these confusing results is that the 293 cells are human embryonic kidney cells which have been transformed by the adenoviral transactivator E1A (Graham *et al.*, 1977). It is possible that expression of this viral transactivator from the 293-based cell lines acted on HSV1 genes which have not been deleted but would not normally be expressed from the disabled viruses. These would be likely to include the IE genes ICP0 and ICP22 (see chapter 6). These proteins would in turn cause the expression of other HSV1 gene products, potentially leading to a situation whereby the viruses could be more toxic to 293 cells than to other target cell types. However, the observed effect on A $\beta$  secretion was specific to the double mutant virus and thus appears to be a direct effect of the expression of both mutant genes in target cells.

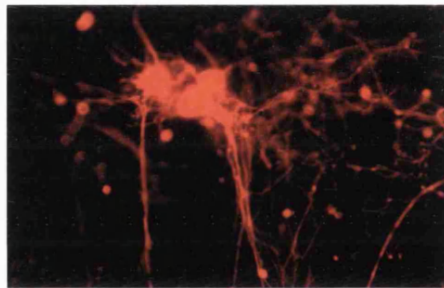
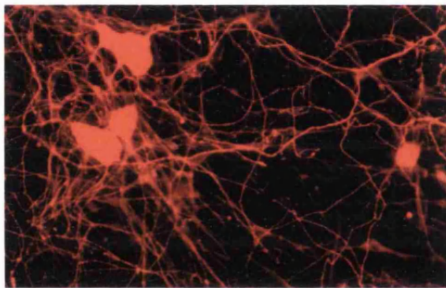
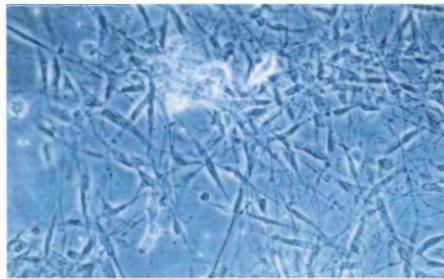
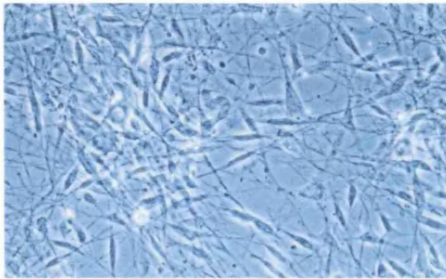
Whether viruses expressing PS1 A246E caused an increase in the ratio of A $\beta$ 1–42 to A $\beta$ 1–40 cannot be assessed here as the levels of A $\beta$ 1–40 are affected by the unexplained phenomenon described above. Furthermore, the sensitivity of the A $\beta$ 1–42 ELISA is very low (it can be seen from figure 8.5.2 that the standard curve for the A $\beta$ 1–42 ELISA showed only very weak immunoreactivity, even when 10ng of peptide was used). This could be attributable to aggregation of the highly fibrillogenic A $\beta$ 1–42 peptide, a problem which is known to often limit the usefulness of this assay (Jill Richardson, Glaxo-Wellcome, personal communication).

No conclusions can be drawn from the experiments described in this section. The ELISAs should be repeated using supernatants from cells other than 293 cells, preferably primary neuronal cultures. The ELISAs should also be repeated using the single APP or PS1 expressing viruses in order to reduce the number of variables being analysed in one experiment. It might also be beneficial to assay the supernatants for soluble APP ( $\alpha$  or  $\beta$ ) to determine which enzyme cleavages had taken place and with approximately what efficiency. However, due to time constraints, these ELISAs have not yet been performed.

## **8.6 Viruses expressing APP Swe and PS1 A246E are more toxic to cultured neurons than viruses expressing APP WT and PS1 WT**

When the 1764 27- P2- 4- *vhs*- APP/PS1 viruses were used to infect non-complementing cells in culture it was noticed that the double mutant virus appeared to be more toxic to cells than the double wild type virus. This observation was also found to extend to primary neuronal cultures, as demonstrated in figure 8.6.1. The figure shows adult rat DRG neurons two weeks post infection at an MOI of 1 with either the double mutant or the double wild type viruses. The top panels show a phase contrast photomicrograph and the bottom panels show the same field of neurons stained with an anti-neuronal specific tubulin antibody (TUJ1), in order to enable clearer visualisation of the neuronal morphology. The panels on the left show neurons infected with the 1764 27- P2- 4- *vhs*- APP WT/PS1 WT virus and the panels on the right show neurons infected with the 1764 27- P2- 4- *vhs*- APP Swe/PS1 A246E virus. It can be seen from the figure that the double wild type virus appears to have caused less toxicity to the target cells than the double mutant virus. The “spiked” processes put out from the neurons infected with the double mutant virus suggests that the supporting cells may have been more susceptible than the neurons themselves to the toxic effects of the APP Swe/PS1 A246E virus.

It would be interesting to repeat this experiment with the full panel of viruses listed in figure 8.3.1. This would determine if the observed toxicity was primarily due to mutant APP or mutant PS1 or if it was a result of an interaction between these two proteins.



1764 27- P2- 4- vhs-  
APP WT/PS1 WT

1764 27- P2- 4- vhs-  
APP Swe/PS1 A246E

**Figure 8.6.1 Adult rat DRG neurons infected with double wild type or double mutant APP and PS1 viruses**

DRG neurons were prepared from an adult rat and infected with the viruses indicated at an MOI of 1. Half the media was changed every third day and the neurons photographed at two weeks post infection. The lower panel shows the same field of view stained with an anti-neuronal specific tubulin antibody (TUJ1) in order to enable clearer visualisation of the neuronal morphology. Details of the antibody staining can be found in section 2.5.7.

In order to try and establish whether cell death was occurring and if so whether this death was proceeding down an apoptotic pathway, infected neurons were analysed for caspase activation.

Caspases are cysteine dependent aspartate proteases which are synthesised as inactive zymogens which are then cleaved during the early stages of apoptosis to release the active form. It has recently been proposed that caspase activation is the primary biochemical event that defines a cellular response as apoptotic (Earnshaw *et al.*, 1999). It has been reported that overexpression of wild type APP causes neurons in culture to degenerate (Nishimura *et al.*, 1998) and that this degeneration involves activation of caspase 3 and is therefore probably mediated via an apoptotic pathway (Uetsuki *et al.*, 1999). As was discussed in chapter 1, it is a matter of some controversy whether PS1 is pro- or anti-apoptotic. The caspase activation experiment was carried out using organotypic hippocampal slice cultures prepared from P7 rat pups. Previous reports on APP WT-mediated caspase activation have successfully used hippocampal neurons and these are obviously more physiologically relevant to AD than DRG neurons. Hippocampal slice cultures were prepared and infected as described in section 2.6.2. Four days post infection the samples were harvested and prepared for caspase assay as described in section 2.5.9. The caspase assay was carried out by Tim Allsopp at the University of Edinburgh. The samples were assayed for activation of caspases with fluorogenic substrates for Grp 1 (caspases 1,4,5,11,12,13,14), Grp 2 (caspases 2, 3, 7) and Grp 3 (caspases 6,8,9 and 10) but no activation was observed in any case (data not shown). Due to the fact that  $1 \times 10^7$  infected neurons were needed for this assay, a time course was not carried out. With hindsight, it might have been sensible to have assayed for activation of fewer caspases, but over a time course.

The fact that the samples were harvested at 4 days post infection means that any activation may have been missed or not yet occurred, especially since the toxicity to the DRG neurons shown in figure 8.6.1 only became apparent after 1-2 weeks post infection. If the caspase experiment was to be repeated, it might also be sensible to include the single APP or PS1 viruses, to preclude the unlikely possibility that the negative result in the case of the double expressing viruses was due to the fact that one protein is pro- and the other is anti-apoptotic. However, due to time constraints this

work has not yet been carried out. Future work will include repeating these infections of primary neuronal cultures and assaying for cell death by a wider range of methods. These should include annexin V surface labelling, propidium iodide staining or looking for DNA fragmentation by terminal transferase dUTP nick-end labelling (TUNEL).

## **8.7 Effects of APP/PS1 on tau phosphorylation**

As discussed in chapter 1, AD is a disease of two pathologies; extracellular A $\beta$  plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau. A major goal in AD research is to identify a link between these two pathologies. The main aim of this thesis was to develop tools by which this might be achieved.

FAD patients with mutations in APP or the presenilins show both increased amyloid deposition and abundant neurofibrillary tangles so it is clear that a mutation in APP or one of the presenilins can cause both pathologies of AD in humans. However, whether A $\beta$ , tau, both or indeed either actually causes the cell death in AD is not known. The situation of both A $\beta$  deposition and tau pathology has not been yet been successfully reproduced in transgenic mice where overexpression of human APP or PS1 mutants causes only the amyloid aspect of the pathology.

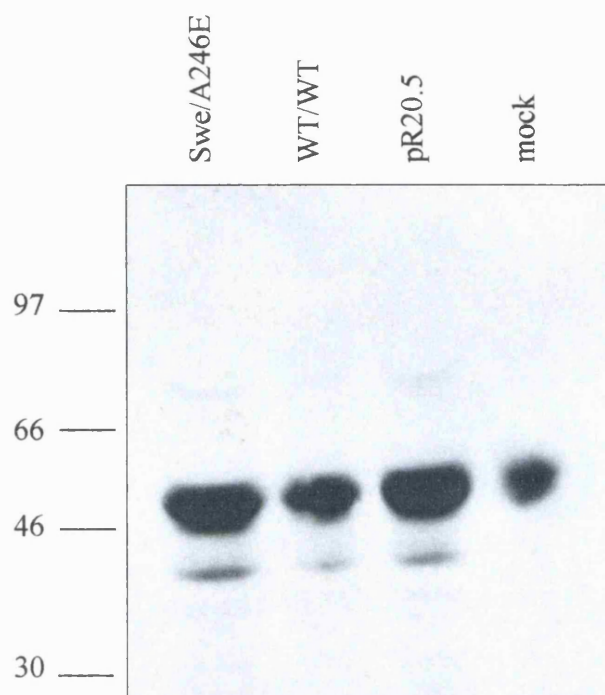
The viruses described in this chapter were used to infect neurons in culture and the neurons were assayed for changes in tau phosphorylation 48 hours post infection. Hyperphosphorylation of tau is well-recognised to be an early event in the formation of neurofibrillary tangles and is often used as a marker for assessing pathological changes in tau function. P7 rat pup organotypic hippocampal slice cultures were prepared and infected as described in section 2.6.2. Neurons were harvested 48 hours post infection and prepared for western blotting as described in section 2.3.1. The western blots were probed with two anti-tau antibodies; TP70 is a phosphorylation insensitive polyclonal antibody and tau-1 is a monoclonal antibody which specifically recognises tau which is unphosphorylated at serine 199 and serine 202. Both antibodies had previously been generated in the Neuroscience department at the Institute of Psychiatry, King's College London.. The western blots of the infected rat hippocampal neurons are shown in figure 8.7.1.

**Figure 8.7.1 Effects of APP WT/PS1 WT and APP Swe/PS1 A246E on tau phosphorylation in rat hippocampal neurons**

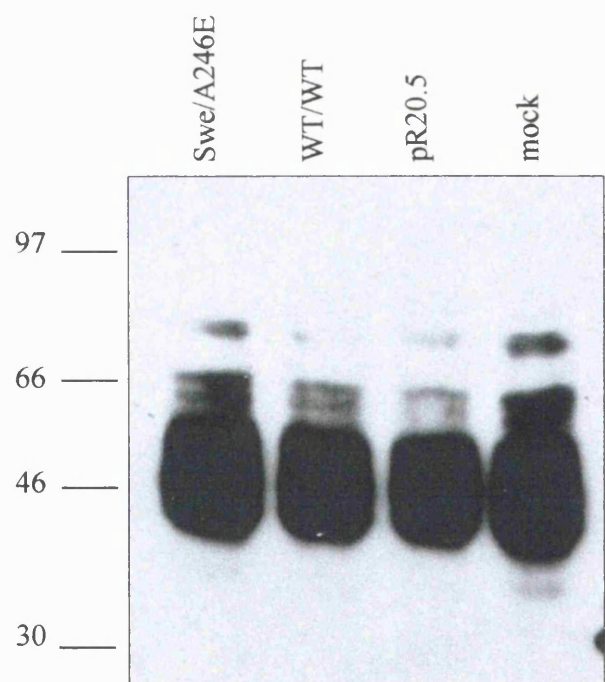
Organotypic hippocampal slices prepared from P7 rat pups were infected with either double mutant, double wild type or negative control (pR20.5) viruses as described in section 2.6.2. 48 hours post infection, the slices were harvested and prepared for western blot analysis as described in section 2.5.1. The western blots were probed with either TP70 (a phosphorylation insensitive polyclonal antibody) or tau-1 (a monoclonal antibody which specifically recognises tau which is unphosphorylated at residues 199 and 201).

Swe/A246E	1764 27- P2- 4- <i>vhs</i> - APP Swe/PS1 A246E
WT/WT	1764 27- P2- 4- <i>vhs</i> - APP WT/PS1 WT
pR20.5	1764 27- P2- 4- <i>vhs</i> - pR20.5
mock	mock infected





**Tau-1**  
(anti-tau species which  
are unphosphorylated at  
residues 199 and 201)



**TP70**  
(anti-total tau)

Figure 8.7.1 shows that overexpression of human APP Swe and human PS1 A246E does not cause any obvious changes in total tau or on the levels of the particular unphosphorylated species detected in rat hippocampal neurons. Any apparent differences between lanes in figure 8.7.1 were confirmed to be attributable to differences in protein loading (data not shown). This result was unsurprising and consistent with many published reports of transgenic animals and cell lines overexpressing mutant APP and PS1 where no changes in tau phosphorylation were seen (Games *et al.*, 1995; Hsiao *et al.*, 1996, Borchelt *et al.*, 1996).

As discussed in section 1.3.5, rodents only express a subset of the tau isoforms implicated in the pathology of human AD. It is therefore possible that rodent tau is not as susceptible to hyperphosphorylation and subsequent tangle formation as human tau. The viral vectors described in this chapter have an advantage over conventional transgenesis in that tau phosphorylation can be assessed in non-rodent species. The vectors can also efficiently transduce primary neurons, cells which are otherwise very hard to transfect. It was therefore planned to repeat the experiment described using primary neuronal cultures from adult marmoset hippocampus and human fetal brain. Ultimately, it was aimed to use these viruses in the adult marmoset brain *in vivo*. Like adult humans, adult marmosets express all six tau isoforms. Although human fetuses only express one of the adult human tau isoforms (0N 3R, no N-terminal inserts and three microtubule binding repeats), this is a different subset to that expressed by rodents (which express 4R tau only). It is therefore possible that primate neurons will provide better models for assessing any APP/PS1 induced tau hyperphosphorylation than rodent neurons.

At the time of writing, the culture system for the adult marmoset hippocampal neurons is still being optimised for viral infection so the tau phosphorylation experiment has not yet been carried out using these neurons. Production of organotypic or dissociated cultures from adult brain tissue is notoriously difficult so it is unsurprising that difficulties have been encountered. However, figure 7.3.1B in the previous chapter demonstrates that human fetal neurons in culture can be efficiently infected by the disabled viruses described in this thesis. The tau phosphorylation experiment has been carried out twice on human fetal neuron cultures and the results are presented in figure

8.7.2. Cultures of human fetal neurons were prepared from aborted fetuses between 12 and 20 weeks gestation by Ritchie Williamson at the Institute of Psychiatry, King's College London. Viral infectivity of the human fetal neuron cultures was found to be variable, despite keeping all known parameters constant. This variability was therefore assumed to be due to the ages of the fetuses, which was different but unknown in each case. It should therefore be noted that for the experiment shown in figure 8.7.2A, approximately 90% of the neurons were infected whereas for the experiment in figure 8.7.2B, the infection efficiency was approximately 40%.

**Figure 8.7.2 Effects of APP WT/PS1 WT and APP Swe/PS1 A246E on tau phosphorylation in human fetal neurons**

Neurons prepared from human fetuses between 12 and 20 weeks gestation were infected with either double mutant, double wild type or negative control (pR20.5) viruses at an MOI of 1. 48 hours post infection, the neurons were harvested and prepared for western blot analysis as described in section 2.5.1. The western blots were probed with either TP70 (a phosphorylation insensitive polyclonal antibody) or tau-1 (a monoclonal antibody which specifically recognises tau which is unphosphorylated at residues 199 and 201). The experiment was repeated twice (A and B in the figure) and in A each infection was performed in duplicate.

A)	WT/WT	1764 27- P2- 4- <i>vhs</i> - APP WT/PS1 WT
	Swe/A246E	1764 27- P2- 4- <i>vhs</i> - APP Swe/PS1 A246E
	pR20.5	1764 27- P2- 4- <i>vhs</i> - pR20.5
B)	Swe/A246E	1764 27- P2- 4- <i>vhs</i> - APP Swe/PS1 A246E
	WT/WT	1764 27- P2- 4- <i>vhs</i> - APP WT/PS1 WT
	pR20.5	1764 27- P2- 4- <i>vhs</i> - pR20.5
	mock	mock infected

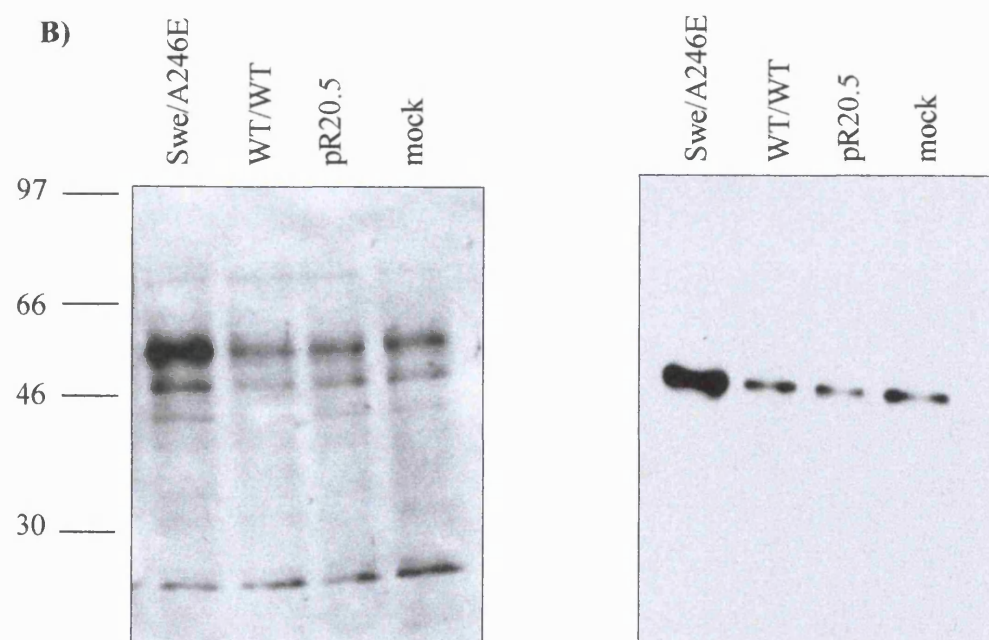
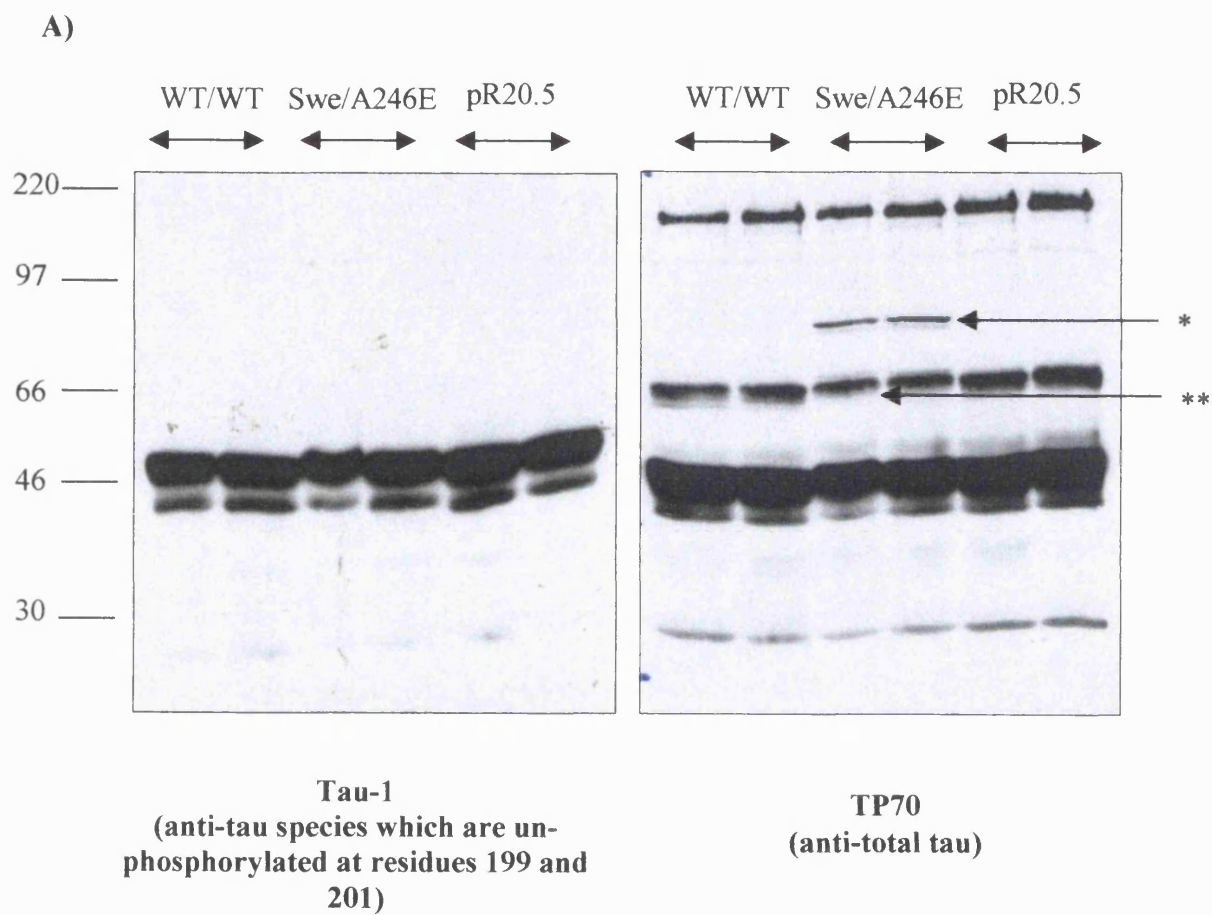


Figure 8.7.2 shows western blots of samples prepared from human fetal neurons infected with either double mutant, double wild type or negative control (pR20.5) viruses. The blots are probed with either TP70 which recognises all tau, regardless of its phosphorylation status or tau-1 which specifically recognises tau which is unphosphorylated at residues 199 and 201. In the first experiment (figure 8.7.2A), it can be seen that there is an extra TP70 immunoreactive band present in the neurons infected with the double mutant virus (indicated by a single asterisk). At around 80kDa, this species is probably too large to be hyperphosphorylated tau but it may be MAP2c, which is known to sometimes cross react with this antibody (Professor Brian Anderton, Institute of Psychiatry, personal communication) although apparently in this case only following infection with the double mutant virus. Alternatively, this species could be a novel protein. The identity of this 80kDa band requires further investigation.

Equally interestingly, there seems to have been a small increase in the molecular weight of a TP70 immunoreactive species which is migrating just below the predominant band at 66kDa in the case of human fetal neurons infected with double wild type or negative control viruses but just above it in the case of the neurons infected with the double mutant virus (the apparent shift is indicated by a double asterisk). This molecular weight shift (a few kDa) is approximately what would be expected as a result of hyperphosphorylation of tau. It would therefore be appealing to conclude that in just 48 hours, the overexpression of APP and PS1 mutants had caused a change in tau phosphorylation in the case of fetal human but not rodent neurons. However, a potentially important caveat to this experiment is that approximately 36 hours after harvesting the samples a low level bacterial contamination became apparent in wells infected with the double mutant virus (some of which were being kept for a longer time point). Although there was no evidence of contamination at the time of harvesting, it is a possibility that bacterial contamination may have affected this result.

When the experiment was repeated, the infection efficiency was significantly lower than for the first experiment (approximately 40% as opposed to approximately 90%). Also, in this second experiment, the TP70 western blot was not very successful (see figure 8.7.2). This potentially interesting observation therefore requires further repetition before any conclusions can be drawn. If the finding can be repeated, it will be

particularly interesting to probe the blots with a range of antibodies recognising differently phosphorylated forms of tau. A shortage of human fetal neurons has so far prevented this experiment being repeated for a third time.

## 8.8 Discussion

This chapter has described the construction of a panel of disabled viruses expressing APP WT or Swe and PS1 WT or A246E either singly or in a number of combinations. Western blots demonstrated that the viruses direct the expression of APP and/or PS1 as appropriate.

A potentially interesting observation on A $\beta$  secretion from HEK293 cells stably overexpressing APP Swe was described in section 8.5. It appeared that the double mutant virus completely abrogated the otherwise high secretion of A $\beta$  from these cells. Clarification of this surprising observation awaits the repeat of the experiment including the single mutant viruses and using primary neuronal cultures and other cells rather than HEK293 cells. Preliminary experiments have suggested that the virus expressing APP Swe and PS1 A246E is more toxic to primary neuronal cultures than the equivalent virus expressing wild type alleles of both genes. Future work to define this response will include repeating the experiment with the single mutant viruses and further attempting to determine the mechanisms by which this toxicity is mediated. This could include repeating the caspase assay over a time course or carrying out alternative assays of cell death.

The main application for the viruses described in this chapter is in the study of APP/PS1 gene interactions and potential effects on tau phosphorylation in a non-rodent model. As a preliminary experiment towards this goal, human fetal neurons were infected and assayed for changes in levels of total and unphosphorylated forms of tau. Preliminary work has suggested that some effect on tau can be observed in some circumstances but considerably more work is required before any conclusions can be drawn. However, whilst these results are very preliminary, this experiment does demonstrate that the viruses can be used in approaches to the study of AD which are not easily possible by other means. The viruses direct high level expression of both APP and PS1 and can be used to efficiently transduce non-rodent neurons. When used in the CNS *in vivo*, efficient transduction of primate neurons with both of these genes should be achievable. This is not possible with technology described elsewhere.

Future work will hopefully include the injection of the APP/PS1 viruses directly into the CNS of a live aged marmoset. It has previously been reported that injection of



A $\beta$  directly into the cerebral cortex of an aged rhesus monkey was sufficient to cause changes in tau phosphorylation but no neurofibrillary tangles (Geula *et al.*, 1998). The potential advantage of the viral vector approach is that this insult would be considerably less transient than in the paper by Geula *et al.*, and the gene delivery would be intracellular, therefore representing a situation more similar to AD. It would be a highly significant finding if this continued neurotoxic insult was found to be sufficient to lead to neurofibrillary tangles of tau in the marmoset brain. Due to a necessary animal licence not yet having been approved by the Home Office, this experiment could not be carried out within the time scale of this thesis. In the intervening period, attempts to culture adult marmoset hippocampal neurons and adult human neurons removed during brain surgery are currently underway. It would therefore appear that the viruses described in this chapter or derivatives thereof may in the future be of considerable use in establishing the molecular link between PS1, APP and tau and the relevance of A $\beta$  and tau pathology to the symptoms of AD. Importantly, this work is not limited to rodents and can therefore be performed in cells which are more relevant to the human disease.

## **CHAPTER 9**

### **GENERAL DISCUSSION**

For many years, new research findings on AD appeared to obscure rather than clarify our understanding of the mechanisms of pathogenesis underlying this complex disease. However, the last few years have seen a unifying pattern emerge; all of the known genetic alterations which have been implicated in AD appear to increase the production and/or deposition of A $\beta$ 42 in the brain. This realisation has led to an increased demand for accurate models of the disease.

Although there have been a plethora of transgenic mice produced, these mice are arguably models of amyloid deposition and not of AD. For an animal model to be truly valuable in dissecting pathogenic mechanisms or assisting in the search for therapies, it should reproduce all the biochemical and behavioural aspects of the human disease. By this definition, the AD models published to date are inadequate in that they consistently lack one of the major pathological hallmarks of the human condition, neurofibrillary tangles of hyperphosphorylated tau. It is therefore imperative that new animal models are developed. These should preferably be in a non-rodent species that has an appropriate life span and genetic organisation for modelling a multifactorial age-dependent disease. The disabled herpes virus vectors described in this thesis may aid in the production of such models.

Chapter 3 of this thesis described the construction and purification of a virus deficient in VP16, ICP34.5/ORF P, ICP34.5 ICP27 and ICP4. Due to repeated sequence elements, this virus was unstable and could not be propagated on a large scale. However, the isolation of this virus was an essential prerequisite to the rest of the work described in this thesis as it facilitated the successful construction and screening of a number of stable cell lines which were able to support the effective growth of such multiply disabled viruses. Generation of these cell lines in turn allowed a stable equivalent of the disabled virus to be generated. This virus then served as a versatile backbone for the insertion of cassettes containing single or pairs of genes either directly into the LAT region or into non-LAT sites in the HSV1 genome.

The viruses described in chapter 6 were found to express high levels of reporter genes in non-complementing tissue culture cells *in vitro*. Characterisation of IE gene expression revealed that the subset of deletions incorporated into these viruses was sufficient to prevent the expression of significant amounts of any of the remaining IE

genes, ICP0, ICP22 and ICP47. Comparison of the expression profile of these ICP4 deleted viruses with their ICP4 expressing counterparts indicated that deletion of ICP27 and mutation of VP16 was not sufficient to completely prevent IE gene expression. Furthermore, in these less disabled viruses, gene expression was not linearly dependent on input virus, with a spike of expression occurring at low MOIs. Both of these characteristics would clearly be undesirable in any gene delivery application and highlight the importance of the generation of the fully disabled vector backbone and full characterisation of its expression profile.

The viruses described in chapter 6 did however express significant levels of the IE/E protein ICP6. This may be due to the previously reported sensitivity of the ICP6 promoter to low levels of ICP0 (Desai *et al.*, 1993; Samaniego *et al.*, 1997). Future work should include an analysis of the expression of a number of other E genes, such as *tk*. If it is found that no other E genes are expressed (as would be expected) then the simplest solution would be to delete ICP6 from the vector backbone. Despite this expression of ICP6, the viruses described were found to be non-toxic to both vero cells and primary neurons in culture, even at high MOI. This was in agreement with previously published work suggesting that the contribution of ICP6 to vector cytotoxicity was minimal (Johnson *et al.*, 1994). The most disabled viruses were found to be able to efficiently transduce several types of neurons in culture and the rat CNS *in vivo*. The use of promoter systems comprising elements of the LAT region allowed these viruses to direct the stable expression of transgenes for at least three weeks in neurons both *in vitro* and *in vivo*.

During the work described in chapters 6 and 7, some interesting differences became apparent between the viruses with reporter gene cassettes in the LAT region and the viruses with insertions in non-LAT sites. These included differences in growth in culture and effects on cultured cells *in vitro*. At least some of these differences were found to be attributable to the presence or absence of the LAT P2 region. The significance of this remains unclear although several lines of evidence suggest that subtle differences in the expression levels of ICP0 may be important. These include the finding that the observed differences in toxicity are removed in situations where ICP0 levels would be expected to be even further minimised, i.e. in naturally non-permissive

cells such as neurons. Potential mechanisms for how the LAT P2 region could cause these effects were discussed in chapter 6.

Whether the presence of the LAT P2 region was also either directly or indirectly responsible for observation that pR19-based viruses appear to deliver genes more efficiently to both cultured neurons and the rat CNS *in vivo* remains unknown. In order to investigate this further it would be important to determine the reason for the apparent difference in transgene expression between the 1764 27- P2- 4- *vhs*- pR20.5 virus and the 1764 27- 4- pR19 virus in this context. It is most likely the promoter system utilised in the pR20.5 virus was less active in the long term, but it is also possible that this virus was not retrogradely transported as efficiently as the pR19 virus. There are several experiments which could distinguish between these two possibilities. Firstly, a rat which had been previously injected into the striatum with the 1764 27- P2- 4- *vhs*- pR20.5 virus could be re-injected several weeks later with the 1764 27- 4- pR19GFP virus. The rat could then be killed two days later and the brain sectioned and stained for *lacZ* expression (expressed only by the initially infecting virus in this experiment). If widespread *lacZ* staining was observed, including staining in the substantia nigra, then it could be concluded that the promoter driving *lacZ* expression in the 1764 27- P2- 4- *vhs*- pR20.5 virus had been previously repressed but that this repression was removed by a factor expressed from the 1764 27- 4- pR19GFP virus. Alternatively, the amount of viral DNA present in the substantia nigra of rats injected with either the 1764 27- P2- 4- *vhs*- pR20.5 virus or the 1764 27- 4- pR19GFP virus could be compared. If the levels were the same it would suggest that the 1764 27- P2- 4- *vhs*- pR20.5 virus had been efficiently retrogradely transported but that promoter shut off had occurred.

The level and distribution of transgene expression from the 1764 27- 4- pR19 viruses described in chapter 7 of this thesis suggest that they might be suitable for development for gene therapy of disorders of the nigrostriatal system such as Parkinson's disease. Work in our laboratory has shown that the pR19 cassette expressing TH or GDNF in a less disabled virus (1764 27-) can significantly reduce apomorphine induced turning behaviour in a 6-hydroxydopamine lesioned rat model of Parkinson's disease for at least 16 weeks post injection (Zi-Qun Han, personal

communication). Currently, work is underway to test the more disabled 1764 27- 4- equivalents of these TH/GDNF expressing viruses.

A unifying hypothesis for all the results presented in chapters 6 and 7 could be that a certain level of ICP0 expression is required for the efficient transactivation of heterologous promoters or the removal of an active repression of such promoters by an unidentified cellular factor. The presence of the endogenous LAT P2 regions could mean that this threshold level of ICP0 is achieved by pR19-based viruses but not by pR20.5-based viruses. This might be due to the LAT P2 region acting as an enhancer on the ICP0 promoter. Alternatively, expression of a protein from the 2kb LAT ORF (Thomas *et al.*, 1999) could compensate for a lack of ICP0 in the pR19-based but not the pR20.5 based viruses. An interesting future experiment to explore this latter possibility would be to introduce a stop codon into the 2kb LAT ORF of the pR19-based viruses and observe if this caused them to assume the growth, toxicity and expression profiles of the pR20.5-based viruses.

In order to establish if ICP0 is indeed involved in mediating the observed differences between the two sets of viruses, ICP0 mRNA levels could be assessed by northern blotting. Alternatively, nuclear runon transcription assays could be carried out in order to compare the rate of transcription of the ICP0 gene in the LAT P2 containing and LAT P2 deleted viruses. If it was found that the presence of a copy of the LAT P2 region in close proximity to the ICP0 gene could enhance the transcription rate of this gene, it would suggest that LAT P2 was acting as an enhancer. Although the problem of quantifying the input virus (see section 6.14) still remains, a rate of transcription rather than an absolute amount of protein might provide a more accurate measure of expression levels. Furthermore, the potential problem of ICP0 protein carryover in the tegument of the virion would be removed. If it was found that the above hypothesis was correct and that a certain level of ICP0 was required for efficient levels of transgene expression, then the pR20.5-based viruses could be adapted to express slightly more ICP0. This could potentially be achieved by using an inducible method such as the “tet-on” or “tet-off” system to drive the expression of ICP0 (Bujard and Gossard, 1992). These systems offer a dose-responsive induction of gene expression and have been successfully used in a number of viral vector systems (Harding *et al.*, 1998; Ho *et al.*,

1996). If a low level of the transactivator was used, a low level of ICP0 would be expressed. Ideally, this level would cause toxicity to the target cell but would be sufficient to overcome the proposed active repression of the promoter driving the expression of the transgene. The fact that such a balance is achievable, in neurons at least, is suggested by the characteristics of the 1764 27- 4- pR19 viruses described in this thesis.

Unfortunately, the promise of gene therapy and the availability of research funding often forces the premature application of gene delivery technologies. The work reported in this thesis demonstrates the need for careful design and optimisation of the vector backbone before the insertion of genes of interest can sensibly occur. As a result of this strategy, the results presented with the viruses containing APP and PS1 are preliminary. However, it was clearly demonstrated that the panel of viruses described in chapter 8 facilitate the high level expression of both APP and PS1. Furthermore, a pronounced but as yet uncharacterised effect resulted from the simultaneous overexpression of mutant versions of these proteins, both on stably transformed 293 cells and on primary neurons in culture.

One of the main aims of this thesis was to express human APP and PS1 in non-rodent neurons. Attempts to culture adult marmoset and human brain biopsy material are currently underway and these efforts were described in chapter 8. The culturing of human fetal tissue is routinely carried out at the Institute of Psychiatry. Using these human fetal neuron cultures, an effect of mutant APP and PS1 on phosphorylation of tau was observed. Although these results were preliminary and the experiment must be repeated, it would be an interesting finding if the overexpression of APP and PS1 mutants caused a reproducible change in tau phosphorylation in the case of neurons from human but not rodent origin. In light of the results described in chapters 7 and 8 of this thesis (discussed above), it might be a necessary future direction to clone APP and/or PS1 WT and mutants into the pR19 cassette, using an IRES to facilitate the simultaneous expression of multiple genes. The resulting viruses might afford more reproducible expression levels *in vivo*. Future work would ideally involve direct injection of the mutant APP/PS1 expressing viruses into the marmoset striatum. However, as was mentioned in chapter 8, animal license restrictions are currently

preventing this experiment from taking place. There are several recently published reports of transgenic mice overexpressing one or more of the tau isoforms present in the adult human brain (Duff *et al.*, 2000; Probst *et al.*, 2000; Spittaels *et al.*, 1999; Ishihara *et al.*, 1999). It would be an interesting future experiment to inject the APP/PS1 viruses (possibly in the pR19 cassette) into the CNS of these human tau transgenic mice. It would be a significant finding if the presence of human APP and/or PS1 mutants expressed by the viruses and the human tau expressed by the mice were sufficient to reproduce both the amyloid and neurofibrillary pathology characteristic of human AD.

The disabled herpes virus vectors are an adaptable system which could, in the future, be of use in delivering factors designed to produce a therapeutic benefit in individuals with AD. Potential approaches could include the delivery of protective ApoE alleles, antisense oligonucleotides to decrease the activity of the secretase enzymes, or specific trophic factors which would prolong neuronal survival. This latter example has been explored in the context of a fimbria fornix lesioned rat, a lesion which disconnects the cholinergic neurons of the medial septum from their NGF supply (reviewed in Cassel *et al.*, 1997). Exogenous replacement of NGF in this model can prevent cholinergic neurons from degeneration and ameliorates some forms of memory deficits. This observation was extended to examine the effects of implantation of primary fibroblasts modified to produce NGF into the brains of aged, cognitively impaired rats and it was found that these rats had increased numbers of NGF receptor-positive neurons in the basal forebrain and they performed better in learning tasks than the controls (Dickinson-Anson *et al.*, 1998). It has also been reported that NGF delivered to the medial septum by a recombinant AAV vector is able to protect cholinergic neurons from fimbria-fornix lesion-induced degeneration (Mandel, 1999). Other putative attempts at 'gene therapy' for AD have targeted the replacement or replenishment of deficient neurotransmitters, for example acetylcholine (ACh). Fibroblasts which were retrovirally transduced to produce ACh have been implanted into the cortex of a lesioned rat and have been reported to ameliorate cognitive dysfunction in this model (Winkler *et al.*, 1995).



Although it might be a real possibility in the future, gene therapy for AD cannot be logically designed until the underlying mechanisms of the pathogenesis are understood. Importantly, the herpes virus vectors described in this thesis might also facilitate the identification of novel targets for treatment by traditional pharmaceutical means.

Despite the advances made in AD research over the past few years, the basic disease processes are still only superficially understood. The stable and non-toxic vector viruses described in this thesis are a powerful and adaptable system which are now potentially well-placed to provide novel insights into the mechanisms of pathogenesis of one of the Western world's most devastating neurological diseases.

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# Equine Herpesvirus 1 Gene 12 Can Substitute for *vmw65* in the Growth of Herpes Simplex Virus (HSV) Type 1, Allowing the Generation of Optimized Cell Lines for the Propagation of HSV Vectors with Multiple Immediate-Early Gene Defects

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Herpes simplex virus (HSV) has often been suggested for development as a vector, particularly for the nervous system. Considerable evidence has shown that for use of HSV as a vector, immediate-early (IE) gene expression must be minimized or abolished, otherwise such vectors are likely to be highly cytotoxic. Mutations of *vmw65* which abolish IE promoter transactivating activity may also be included to reduce IE gene expression generally. However, when *vmw65* mutations are combined with an IE gene deletion, such viruses are hard to propagate, even on cells which otherwise complement the IE gene deletion effectively. We have found that *vmw65* mutants can be effectively grown on cell lines expressing equine herpesvirus 1 gene 12, a non-HSV homologue of *vmw65* with little sequence similarity to its HSV counterpart. This prevents repair by homologous recombination of *vmw65* mutations in the virus, which would occur if mutations were complemented by *vmw65* itself. The gene 12 protein is not packaged into HSV virions, which is important if viruses grown on such cells are to be used as vectors. These results not only further strengthen the evidence for direct functional homology between and similar modes of action of the two proteins but have allowed the generation of gene 12-containing cell lines in which ICP4 and ICP27 expression is induced by virus infection (probably by ICP0) and which give efficient growth of viruses deficient in ICP27, ICP4, and *vmw65* (the viruses also have ICP34.5/ORFP deleted). Efficient growth of such viruses has not previously been possible. As these viruses are highly deficient in IE gene expression generally, such virus-cell line combinations may provide an alternative to HSV vectors with deletions of all four of the regulatory IE genes which, for optimal growth, require cell lines containing all four IE genes but which are hard to generate due to the intrinsic cytotoxicity of each of the proteins.

Herpes simplex virus (HSV) type 1 (HSV-1) has often been suggested as a vector for gene delivery to the nervous system and also other cell types (for reviews, see references 4 and 13). As a vector, HSV has a number of potential advantages in that it naturally enters latency in neurons, providing the possibility of long-term gene expression; does not integrate into the host genome, preventing insertional mutagenesis (for example, the activation of oncogenes or inactivation of tumor suppressor genes); can accept very large DNA insertions, allowing the delivery of multiple genes; is easy to propagate; and can infect a wide variety of other cell types, as well as neurons. Some of these cell types are hard to transduce by other means. In our studies, for example, this has allowed highly efficient gene delivery to dendritic cells (>70%) and less efficient delivery to CD34<sup>+</sup> hemopoietic cells (≈10%; 6), cell types which are otherwise both generally recalcitrant to gene transfer.

However, while HSV-1 is highly prevalent in the human population, in the vast majority of cases giving no obvious signs of disease, for use as a vector, the virus must be disabled for safety and to minimize toxicity to target cells. Various strategies for disablement have been reported, including the removal of genes which are unnecessary for growth *in vitro* but necessary for pathogenesis *in vivo*. Such genes include those encod-

ing thymidine kinase (18, 26), ribonucleotide reductase (15), and ICP34.5 (5). However, for minimal toxicity, it has become apparent that expression of the regulatory immediate-early (IE) genes ICP0, ICP4, ICP22, and ICP27, which are themselves cytotoxic, must be minimized (20–22, 35, 43). Such reductions in IE gene expression minimize transcription from the vast majority of the 80 or so other genes in the HSV genome. Removal of ICP4 or ICP27 from the HSV genome completely prevents virus growth, and so, such deletions must be complemented in the cells used for virus propagation (9, 31). Deletion of ICP22 and/or ICP0, while these genes are not absolutely essential for virus growth (28, 32, 37, 42), reduces the virus titer. Thus, for the growth of HSV mutants with multiple IE gene deficiencies, cell lines must be produced which effectively complement deletions from the virus, and for effective growth of viruses with deletions in ICP4, ICP27, ICP22, and ICP0, all of these deficiencies would optimally need to be complemented. However, as the IE proteins are highly cytotoxic (20), IE gene expression in cell lines must be tightly regulated. This is usually achieved by the use of the homologous IE gene promoters, which are relatively inactive in the absence of virus infection (e.g., E5 cells [ICP4], B130/2 cells [ICP27], E26 cells [ICP4 and ICP27], and FO6 cells [ICP4, ICP27, and ICP0]; 10, 19, 34, 36). This reduces the problem of IE protein cytotoxicity but still leaves an inherent problem in the generation of cells which are highly effective at complementing multiple IE gene deficiencies.

A second strategy to reduce IE gene expression, rather than deletion of the IE genes themselves, is to include mutations in

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the gene encoding vmw65. vmw65 is a virion protein which transactivates IE promoters after virus infection (2, 27), and while it is an essential structural protein, specific mutations abolish the transactivating capability of the protein without affecting the structural integrity of the virus (1, 39). These mutations vastly reduce IE gene expression although at high multiplicity or, with the inclusion of hexamethylene bisacetamide (HMBA) in the medium, still allow efficient virus growth in culture (25).

For the construction of vector viruses, we have thus taken the approach of combining mutations in vmw65, which should reduce the expression of all IE genes, with deletion of ICP27 and/or ICP4, the two essential IE genes, giving viruses as described above, in which overall IE gene expression is minimized. However, we have found that in combination with deletion of ICP27 and/or ICP4, growth of vmw65 mutants is vastly reduced, even with HMBA and even in cells which otherwise effectively complement the deficiencies in ICP27 and/or ICP4. As in vmw65-deficient viruses the gene encoding vmw65 has not been deleted, with in our case only a small insertion in the protein (the *in1814* mutation; 1), unaltered vmw65 cannot be included in the cells for virus growth. Here, homologous recombination between virus DNA and the vmw65 gene in the cell line would repair the vmw65 defect, preventing the stable propagation of viruses with the mutation. Moreover, such viruses would then package fully functional vmw65 derived from the cell line, reducing the effects of the mutation when the viruses were used as vectors in noncomplementing cells.

To solve this problem, we have tested the novel approach of using a non-HSV homologue of vmw65 (from equine herpesvirus 1 [EHV-1]) for complementation of vmw65-mediated defects in virus growth. The EHV-1 vmw65 homologue (gene 12) (29) has previously been shown by cotransfection experiments to be capable of transactivating HSV IE promoters (29), suggesting that the approach is possible. We have found that while there is minimal DNA similarity between EHV-1 gene 12 and the HSV gene encoding vmw65 (46% identity overall), minimizing the likelihood of repair of vmw65 defects by homologous recombination, when EHV-1 gene 12 is constitutively expressed in the cells used for virus propagation, growth defects associated with vmw65 mutation are abolished. Importantly, EHV-1 gene 12 protein is not packaged into HSV virions grown on such cells, and so, when viruses are used on noncomplementing cells, IE gene deficiencies associated with vmw65 mutation will be retained. When EHV-1 gene 12 is included in cells together with ICP4 and/or ICP27, viruses with ICP4 and/or ICP27 deleted and with mutations in vmw65 can be effectively propagated, which was not previously possible, although here the choice of the promoter driving ICP4 and/or ICP27 is important.

#### MATERIALS AND METHODS

**Cell culture and virus propagation.** The cell lines used were all based on BHK cells and cultured in Dulbecco's modified Eagle medium plus 10% fetal calf serum. Cell lines were generated by using standard methods of calcium phosphate transfection with the plasmids indicated, antibiotic selection, and cloning out of resistant colonies under constant selection. Neomycin was used at a concentration of 800 µg/ml, and phleomycin D1 (Zeocin; Cayla, Toulouse, France) was used at a concentration of 750 µg/ml. Where indicated, HMBA was included in the medium at a concentration of 3 mM and dexamethasone was used at a concentration of 1 µM. Growth curves were determined in duplicate by using 24-well plates at a multiplicity of infection (MOI) of 0.01. The yields presented are average values per well. All tissue culture reagents were from GIBCO unless otherwise stated. Chloramphenicol acetyltransferase (CAT) assays were performed by standard methods (16) using HSV IE promoter constructs pIGA102, pIGA95, and pIGA65 encoding the ICP4, ICP27, and ICP0 promoters driving *cat* (14; gift from S. Silverstein, Columbia University, New York, N.Y.) and pCMV-VP16 containing HSV vmw65 under cytomegalovirus (CMV) promoter control (6).

**Viruses.** The viruses used in this study are shown in Fig. 1. Virus strains *in1814* and 1764 have been previously reported (1, 5). Virus strains 17+/MSVlacZ/43 and 17+/27-/MSVlacZ/43 contain a Moloney murine sarcoma virus long terminal repeat (11) promoter/*lacZ* (*Hind*-*Bam*HI from pCH110; Pharmacia) cassette inserted into the unique *Nsi*I site of the nonessential UL43 gene (24) of HSV-1 strains 17+ (wild type) and 17+/27-w, respectively. HSV strains 17+/27-w and 1764/27-w were prepared by removing the *lacZ* insertion from the ICP27 gene in virus strains 17+/27-/pR20 and 1764/27-/pR20 (see below), respectively, by recombination with empty ICP27 flanking regions and selection of non-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-staining recombinant plaques using B130/2 cells which complement ICP27 (19). Virus strains 17+/27-/pR20 and 1764/27-/pR20 contain a latency-associated transcript (LAT) (nucleotides [nt] 118,866 to 120,219 [*Pst*I-*Bst*XI])/CMV/*lacZ* cassette inserted so as to delete the entire ICP27 coding sequence, UL55, UL56 (both nonessential genes; 30), and part of the LAT region in virus strains 17+ and 1764, respectively, using flanking regions (nt 110,095 to 113,229 [*Eco*RI-*Nde*I] and 120,468 to 125,068 [*Hpa*I-*Sac*I]) separated by a unique *Bgl*II site in plasmid pΔ27LAT and the selection and purification of X-Gal-staining plaques on B130/2 cells. Virus strain 1764/27-/4-/pR20.5 was constructed by insertion of a cassette consisting of the gene for green fluorescent protein (E-GFP; Clontech) and *lacZ* driven by the CMV and Rous sarcoma virus promoters, respectively, in a back-to-back orientation and separated by LAT sequences (*Pst*I-*Bst*XI as described above) into ICP4 flanking regions (nt 123,459 to 126,774 [*Sau*3A1-*Sau*3A1] and 131,730 to 134,792 [*Sph*I-*Kpn*I]), with nt 124,945 to 125,723 [*Nor*I-*Nor*I; encodes ICP34.5] deleted, separated by unique *Xba*I and *Sal*I sites in plasmid pΔICP4 and recombination into virus strain 1764/27-w by using B4/27 cells, which complement both ICP27 and ICP4. X-Gal-staining, green fluorescent plaques were selected and further purified. Cell line B4/27 was prepared by cotransfection of pSG130BS [containing the ICP27 promoter, coding sequence, and poly(A); 38]; p4/2, which contains the ICP4 promoter, coding region, and poly(A) (nt 126,764 to 131,730 [*Dde*I-*Sph*I]) inserted into pSP72 (Promega); and pMAMneo (Invitrogen) into BHK cells. Neomycin-resistant clones were then selected. Virus structures were checked by Southern blotting and also by Western blotting with anti-ICP27 and anti-ICP4 antibodies (see below) of the viruses on noncomplementing cells, showing ICP27 and/or ICP4 not to be expressed from the corresponding viruses (data not shown). A full characterization of the more disabled of the viruses will be presented elsewhere. All nucleotide numbers refer to the HSV-1 strain 17+ genomic sequence (GenBank file *hclp*).

**Plasmids.** For the ICP27- and EHV-1 gene 12-containing cells generated in this study, EHV-1 gene 12 was inserted into pcDNA3 (Invitrogen) between the *Eco*RV and *Xba*I sites following excision of the EHV-1 gene 12 coding sequence from plasmid pcDNA/AmpETIF (supplied by M. Grapes, Marie Curie Institute) with *Eco*RI and *Xba*I. The ICP27 coding sequence promoter and poly(A) (excised from pSG130BS with *Sac*I and *Sph*I) were inserted between the *Eco*RI and *Sal*I sites in pGKneo (40), thus putting each of the genes into a plasmid encoding neomycin selection. For the generation of cell lines containing ICP4, the following plasmids were constructed. For ICP4 under ICP4 promoter control, a phleomycin resistance gene cassette was excised from plasmid pVgRΔR (Invitrogen) as a *Bam*HI fragment and inserted into the unique *Bgl*II site of plasmid p4/2 (see above), giving plasmid p4/2zeo. For ICP4 under ICP27 promoter and poly(A) control, the ICP4 promoter in plasmid p4/2zeo (upstream of the *Bst*EII site [HSV nt 131,187]) was replaced with a *Bam*HI-*Drd*I (HSV nt 113,322 to 113,728) promoter fragment from pSG130BS. The ICP4 poly(A) sequence was replaced by removal of sequences after the *Mse*I site (HSV nt 127,167), which were replaced with an *Eco*NI-*Sac*I (HSV nt 115,267 to 115,743) fragment from pSG130BS encoding the ICP27 poly(A) sequence. For mouse mammary tumor virus (MMTV) promoter control, the neomycin resistance gene (excised as a *Bam*HI fragment) in plasmid pMAMneo (Invitrogen) was replaced with the phleomycin resistance gene as described above, again as a *Bam*HI fragment. The ICP4 coding region (nt 127,167 to 131,187 [*Mse*I-*Bst*EII]) was then inserted after the MMTV promoter at the *Xho*I site, giving plasmid pMAMzeo/ICP4. All plasmids were linearized for generation of cell lines.

**Western blotting.** Western blotting was performed on cell extracts or virus stocks by standard methods using ECL (Amersham) and antibodies to either HSV-1 ICP27, ICP4, ICP0 (all from ABI) or vmw65 (POS1; supplied by Matt Grapes) or EHV-1 gene 12 (23). Equal loading of lanes was checked by Coomassie blue staining of duplicate gels, in most cases using extract from  $\approx 10^5$  cells per lane or  $10^5$  PFU of virus (HSV or EHV) per lane.

#### RESULTS

**EHV-1 gene 12 can transactivate HSV IE promoters.** Previous work has shown that a non-HSV homologue of vmw65, EHV-1 gene 12, can transactivate at least some HSV IE promoters (29). Other herpesviruses encode similar homologous proteins. For example, the genomes of varicella-zoster virus, bovine herpesvirus 1, and EHV-4 also encode homologues of vmw65 (3, 8, 29). Of these proteins, the EHV-1 gene 12-encoded protein has been shown to have the greatest transac-

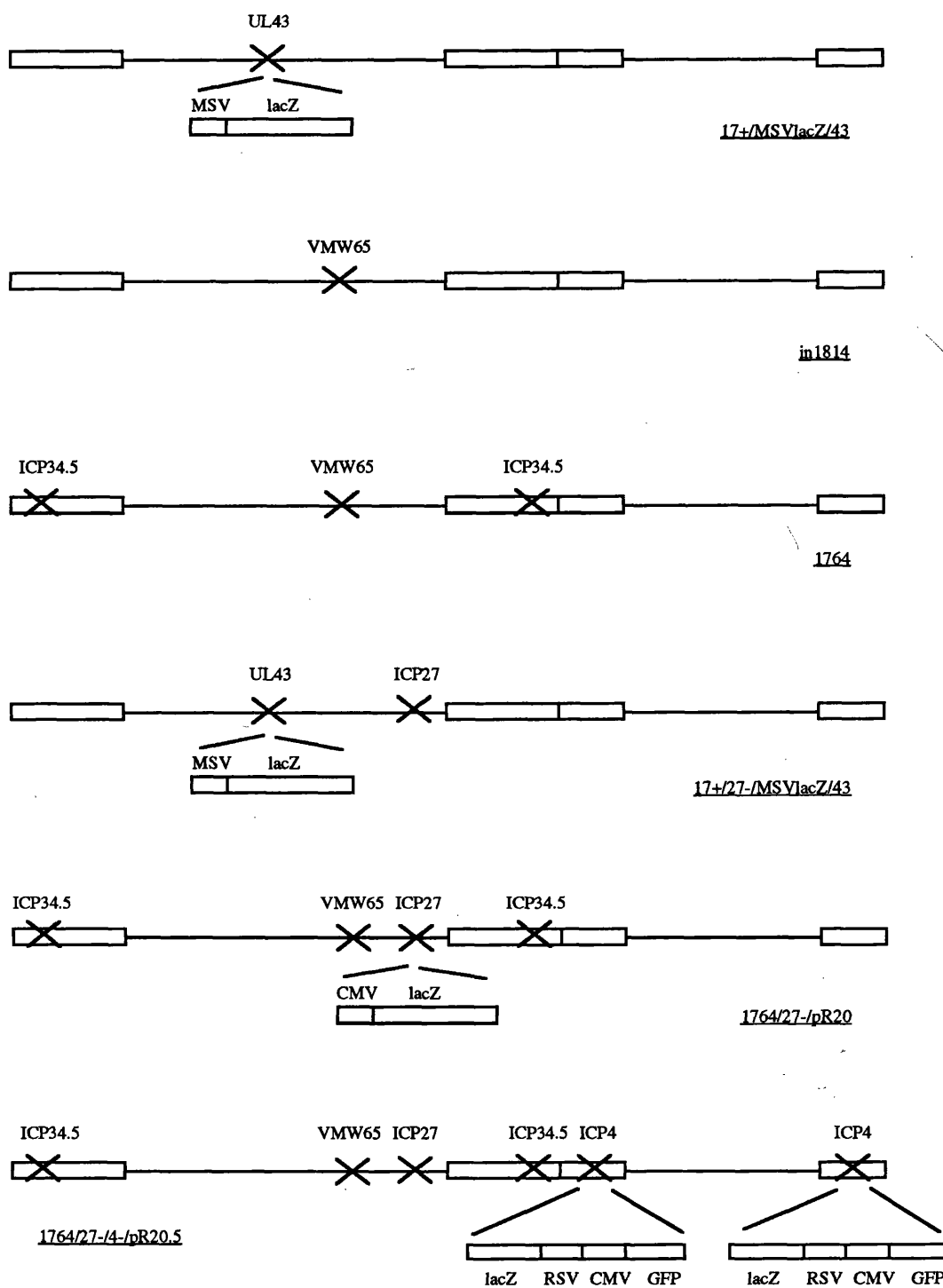


FIG. 1. Viruses used in the study. Further details are given in Materials and Methods. RSV, Rous sarcoma virus; GFP, green fluorescent protein; MSV, Moloney murine sarcoma virus.

tivating effect on HSV IE promoters, at least on the promoters for the ICP4- and ICP0-encoding genes (29). In an initial experiment using the HSV ICP0, ICP4, and ICP27 promoters driving a *cat* reporter gene in cotransfection experiments, we sought to confirm and further extend this work to the ICP27 promoter, which had not previously been tested. These results showed efficient transactivation of ICP0 and ICP4 (similar to

the transactivation provided by HSV vmw65), as before, but minimal effects on the ICP27 promoter (Fig. 2). Thus, EHV-1 gene 12 may be able to complement the growth of HSV deficient in vmw65 transactivating activity, as well as to function in cotransfection experiments as reported previously and here, although it might be expected that some deficiency in ICP27 would still be apparent. However, after activation of ICP0, it



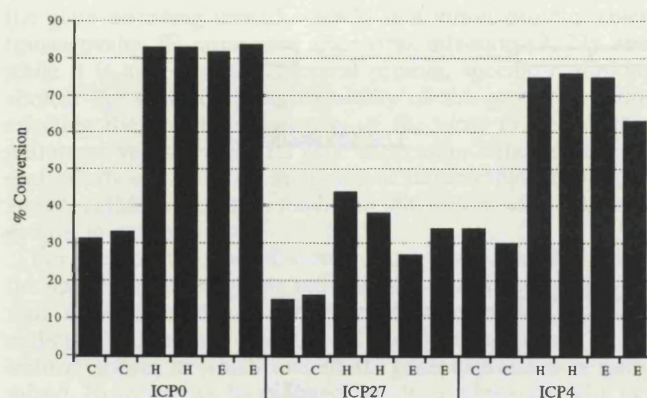


FIG. 2. EHV-1 gene 12-induced transactivation of HSV IE promoters. Duplicate CAT assays were performed in which plasmids encoding the HSV-1 ICP0, ICP27, and ICP4 promoters driving *cat* (see Materials and Methods) were co-transfected into BHK cells together with either a vector control plasmid (C), a plasmid with HSV-1 vmw65 under CMV promoter control (H), or a similar plasmid containing EHV-1 gene 12 (E). Percent conversion of the substrate to the acetylated form is shown.

might also be expected that any ICP27 deficiency would be minimal due to the likely potential of ICP0 to transactivate the ICP27 promoter (see later).

**EHV-1 gene 12 complements growth deficiencies in HSV-1 vmw65 mutants.** HSV vmw65 mutants deficient in transactivating activity but not packaging, such as *in1814* or V422 (1, 39), can be grown in noncomplementing cells at a high MOI or with inclusion in the medium of HMBA (25), which has a generalized promoter-activating effect. However, we have found that when such mutants have further disabling mutations, for example, in ICP27, even in cells which effectively complement such deficiencies, only limited growth occurs. This is the case even at a high MOI or with HMBA. To test whether EHV-1 gene 12 could overcome such growth deficiencies, EHV-1 gene 12 was subcloned into pcDNA3 (Promega) containing a neomycin resistance gene and driving gene 12 from a CMV promoter. This was transfected into BHK cells either alone or at an equal molar ratio with a second plasmid containing the HSV ICP27 promoter, coding sequence, and poly(A) region. After neomycin selection, the contents of the wells were trypsinized and reseeded into new six-well plates and the ability to support the replication of viruses (as estimated by total virus yield) with an inactivating mutation in vmw65 (*in1814*) or with an inactivating mutation in vmw65 together with deletion of ICP34.5 (1764) or also together with ICP27 (1764/27–pR20) was tested. The viruses used for the work described in this paper are shown in Fig. 1.

The experiments described above were performed both in the presence and in the absence of HMBA, giving, in these uncloned, neomycin-resistant cells, the average effect of EHV-1 gene 12 expression without the clonal variation which could result from picking of individual colonies. The experiments were performed at a relatively low MOI, as growth deficiencies associated with mutations in vmw65 are more evident under these conditions. Figure 3A shows that in each case, gene 12 considerably improved virus growth and minimized the difference in virus yield obtained when HMBA was included in the medium (similar to the case of the wild-type virus, where the effect of HMBA is minimal). This shows that EHV-1 gene 12 can functionally compensate for deficiencies in virus growth caused by vmw65 inactivation and allow consid-

erable improvement in the growth of vmw65/ICP27 double mutants.

Next, cell lines were cloned out after transfection with only the ICP27 gene-containing plasmid or the ICP27 gene-containing plasmid together with the EHV-1 gene 12-containing plasmid. This again showed that in most cases, better growth of viruses deficient in both ICP27 and vmw65 could be obtained by using clones resulting from the dual transfection (Fig. 3B shows five representative clones in each case). Clones A5 and B6 were used in further experiments in comparison to untransfected BHK control cells. These experiments showed considerably larger plaques when viruses with vmw65 inactivated, with or without deletion of ICP27, were grown on cells containing EHV-1 gene 12. EHV-1 gene 12 expression was confirmed in both the uncloned cells (Fig. 4A) and the cloned cells, where it was found that significant EHV-1 gene 12 could only be detected after virus infection (longer exposures showed expression also without infection; Fig. 4B). This suggests that EHV-1 gene 12 is toxic to cells and selected against such that expression is induced by, e.g., ICP0 after infection. The anti-EHV-1 gene 12 antibody does not cross-react with HSV vmw65 (see later). One-step growth curves further confirmed the increased permissivity of EHV-1 gene 12-containing cells for the growth of HSV-1 vmw65 mutants (Fig. 5). For both of these experiments (except as noted otherwise), HMBA was included, which only slightly increased virus growth in EHV-1 gene 12-containing cells, further demonstrating the relatively poor growth characteristics of ICP27/vmw65 double mutants, even in the presence of HMBA and when ICP27 is otherwise effectively complemented. The above results show that these growth defects can be minimized by the inclusion of EHV-1 gene 12 in the cells used for virus growth. This is, surprisingly, expressed at relatively low constitutive levels in cloned cells, but expression is induced after virus infection.

**EHV-1 gene 12 is not packaged into HSV virions.** For EHV-1 gene 12 to be useful in cell lines for the growth of vmw65-deficient HSV for vector purposes, it is important that EHV-1 gene 12 cannot be packaged into HSV virions, since if this were the case, the advantages of reduced HSV IE gene expression in target cells in potentially reducing the cytotoxicity of such vectors would be minimal. To test whether EHV-1 gene 12 could be packaged into HSV virions, two experiments were performed. First, one-step growth curves were determined. HSV vmw65 mutants were plated at a low MOI onto nonengineered BHK cells. Growth was identical whether or not virus stocks had previously been prepared on EHV-1 gene 12-containing cells or nonengineered BHK cells (data not shown). If EHV-1 gene 12 was packaged, a growth advantage would have been expected here. Second, Western blotting of virus samples was performed in which vmw65 and vmw65/ICP27 mutants were grown on either BHK cells or cells containing EHV-1 gene 12. These were probed with either an anti-HSV vmw65 antibody or an anti-EHV-1 gene 12 antibody in comparison to a purified EHV-positive control. This showed a strong anti-vmw65 signal in all cases with HSV or the various HSV vmw65 mutants but no signal for EHV-1 gene 12, other than in the EHV-1 positive-control lane, independently of the cell line on which the viruses were prepared (Fig. 6). Thus, EHV-1 gene 12 is not packaged into HSV virions even though it can functionally compensate for growth deficiencies caused by mutations affecting the transactivating activity of vmw65. vmw65 is detectable in these blots, as the *in1814* mutation used is a linker insertion mutation which produces a protein that is capable of fulfilling its essential structural role yet is incapable of transactivating IE gene expression.

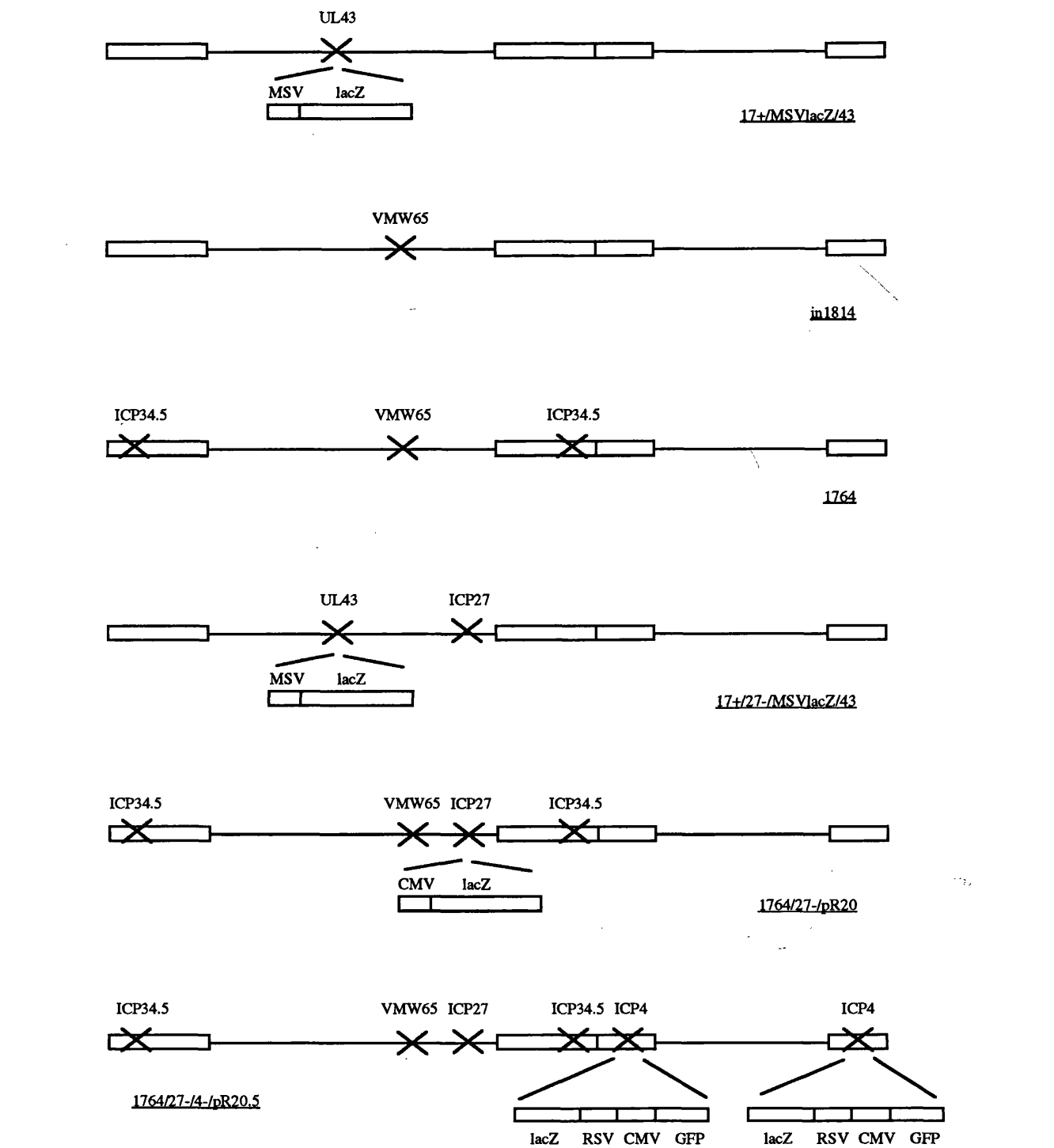


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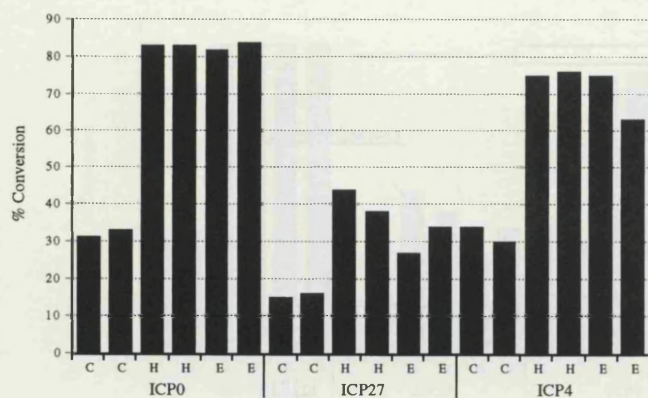


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erable improvement in the growth of *vmw65*/ICP27 double mutants.

Next, cell lines were cloned out after transfection with only the ICP27 gene-containing plasmid or the ICP27 gene-containing plasmid together with the EHV-1 gene 12-containing plasmid. This again showed that in most cases, better growth of viruses deficient in both ICP27 and *vmw65* could be obtained by using clones resulting from the dual transfection (Fig. 3B shows five representative clones in each case). Clones A5 and B6 were used in further experiments in comparison to untransfected BHK control cells. These experiments showed considerably larger plaques when viruses with *vmw65* inactivated, with or without deletion of ICP27, were grown on cells containing EHV-1 gene 12. EHV-1 gene 12 expression was confirmed in both the uncloned cells (Fig. 4A) and the cloned cells, where it was found that significant EHV-1 gene 12 could only be detected after virus infection (longer exposures showed expression also without infection; Fig. 4B). This suggests that EHV-1 gene 12 is toxic to cells and selected against such that expression is induced by, e.g., ICP0 after infection. The anti-EHV-1 gene 12 antibody does not cross-react with HSV *vmw65* (see later). One-step growth curves further confirmed the increased permissivity of EHV-1 gene 12-containing cells for the growth of HSV-1 *vmw65* mutants (Fig. 5). For both of these experiments (except as noted otherwise), HMBA was included, which only slightly increased virus growth in EHV-1 gene 12-containing cells, further demonstrating the relatively poor growth characteristics of ICP27/*vmw65* double mutants, even in the presence of HMBA and when ICP27 is otherwise effectively complemented. The above results show that these growth defects can be minimized by the inclusion of EHV-1 gene 12 in the cells used for virus growth. This is, surprisingly, expressed at relatively low constitutive levels in cloned cells, but expression is induced after virus infection.

**EHV-1 gene 12 is not packaged into HSV virions.** For EHV-1 gene 12 to be useful in cell lines for the growth of *vmw65*-deficient HSV for vector purposes, it is important that EHV-1 gene 12 cannot be packaged into HSV virions, since if this were the case, the advantages of reduced HSV IE gene expression in target cells in potentially reducing the cytotoxicity of such vectors would be minimal. To test whether EHV-1 gene 12 could be packaged into HSV virions, two experiments were performed. First, one-step growth curves were determined. HSV *vmw65* mutants were plated at a low MOI onto nonengineered BHK cells. Growth was identical whether or not virus stocks had previously been prepared on EHV-1 gene 12-containing cells or nonengineered BHK cells (data not shown). If EHV-1 gene 12 was packaged, a growth advantage would have been expected here. Second, Western blotting of virus samples was performed in which *vmw65* and *vmw65*/ICP27 mutants were grown on either BHK cells or cells containing EHV-1 gene 12. These were probed with either an anti-HSV *vmw65* antibody or an anti-EHV-1 gene 12 antibody in comparison to a purified EHV-positive control. This showed a strong anti-*vmw65* signal in all cases with HSV or the various HSV *vmw65* mutants but no signal for EHV-1 gene 12, other than in the EHV-1 positive-control lane, independently of the cell line on which the viruses were prepared (Fig. 6). Thus, EHV-1 gene 12 is not packaged into HSV virions even though it can functionally compensate for growth deficiencies caused by mutations affecting the transactivating activity of *vmw65*. *vmw65* is detectable in these blots, as the *in1814* mutation used is a linker insertion mutation which produces a protein that is capable of fulfilling its essential structural role yet is incapable of transactivating IE gene expression.



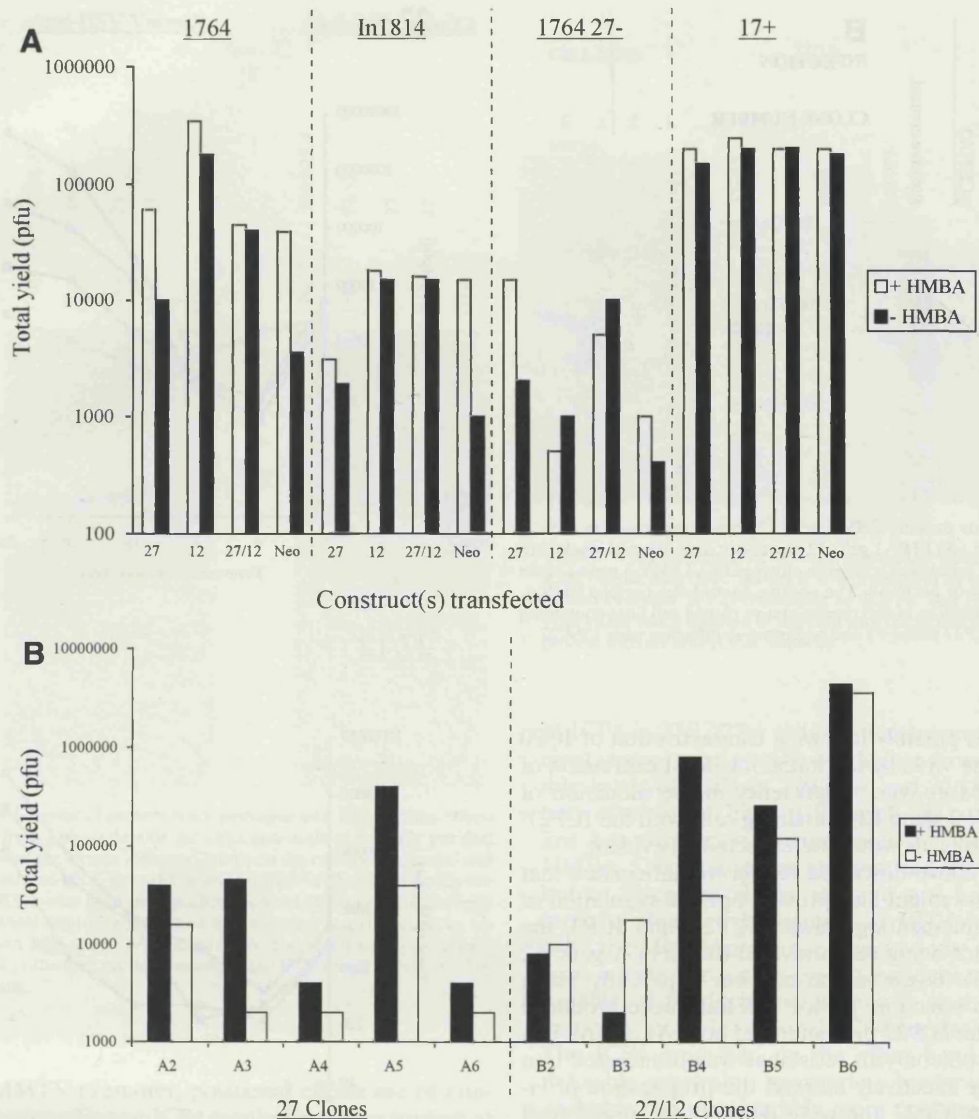


FIG. 3. EHV-1 gene 12 complements growth deficiencies in HSV *vmw65* mutants. (A) Total yields of the indicated viruses when grown on uncloned BHK cells stably transfected with either a control plasmid (Neo), a plasmid encoding ICP27 under ICP27 promoter control (columns 27), a plasmid encoding EHV-1 gene 12 under CMV promoter control (columns 12), or both the ICP27- and EHV-1 gene 12-encoding plasmids together (columns 27/12). (B) Growth of an HSV-1 mutant deficient in both ICP27 and *vmw65* (1764/27-/*pR20*) on individual clones of BHK cells stably transfected with either the ICP27 plasmid or both the ICP27- and EHV-1 gene 12-encoding plasmids as for panel A.

**Promoter choice for IE gene expression is important for cell lines containing EHV-1 gene 12 which also complement multiple HSV IE gene deficiencies.** We were interested in generating cell lines capable of allowing the effective growth of viruses with *vmw65* deficiencies and in which the genes for both ICP27 and ICP4 were also deleted, as such viruses might be expected to be minimally toxic in noncomplementing cells due to anticipated minimal IE gene expression (see introduction). We have found as described above, that the ICP27 promoter driving ICP27 provides effective cell lines complementing viruses with the gene for ICP27 deleted whether or not the cells contain EHV-1 gene 12. Thus, low-level gene 12 expression does not appear to significantly induce the expression of ICP27, which would be expected to be toxic, thus preventing the stable production of such cells. This could be because EHV-1 gene 12 does not significantly transactivate the ICP27 gene promoter (see earlier), but after virus infection, ICP27

expression, like EHV-1 gene 12 expression, is induced, allowing virus growth. This was confirmed by Western blotting of ICP27 and EHV-1 gene 12-containing cells before and after infection with ICP27- and ICP27/*vmw65*-deficient viruses (Fig. 7). ICP27 induction on cells containing ICP27 alone was also tested. The gene for ICP27 is completely deleted from these viruses, so no ICP27 can be expressed and detected from the incoming virus. After probing with an anti-ICP27 antibody, only minimal ICP27 levels could be detected before virus infection in both cases. These levels were greatly increased 24 h after infection with both ICP27- and ICP27/*vmw65*-deficient viruses in ICP27- and EHV-1 gene 12-containing cells and with ICP27-deficient virus on ICP27-containing cells but with a considerably smaller increase in ICP27-containing cells infected with the ICP27/*vmw65*-deficient virus. Thus, both ICP27 gene expression and higher-level EHV-1 gene 12 expression (Fig. 4) are induced by virus infection of ICP27 gene- and EHV-1 gene

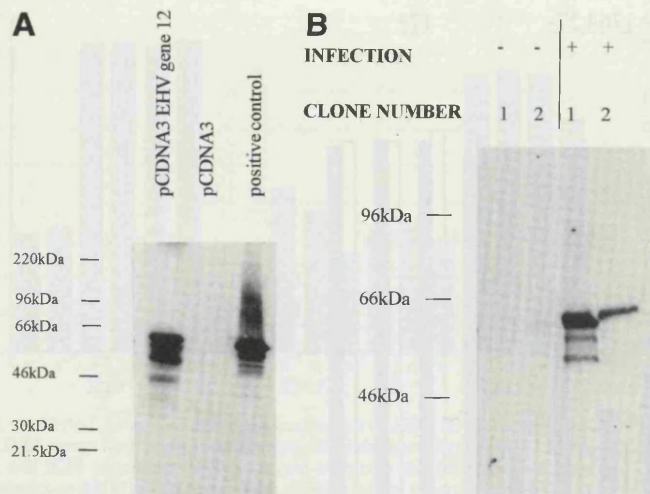


FIG. 4. Western blots showing EHV-1 gene 12 protein expression in EHV-1 gene 12-containing cells. (A) EHV-1 gene 12 expression in uninfected, uncloned-out, neomycin-resistant cells transfected with either pDNA3 EHV-1 gene 12 (see Materials and Methods) or pCDNA3. The positive control was purified EHV-1. (B) EHV-1 gene 12 expression in two representative cloned cell lines containing EHV-1 gene 12 and ICP27 with (+) and without (–) infection with 1764/27–/pR20.

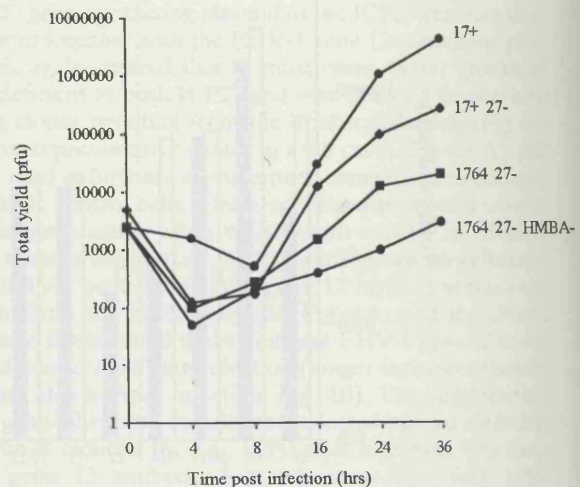
12-containing cells, possibly following transactivation of ICP0 expression from the virus by the initial low-level expression of EHV-1 gene 12. Moreover, a deficiency in the induction of ICP27 in non-EHV-1 gene 12-containing cells with the ICP27/vmw65-deficient virus, as would be expected, is evident.

Thus, from the above-described results we anticipated that the ICP27 promoter might also provide optimal regulation of ICP4 in cells complementing vmw65, ICP27, and ICP4, the ICP27 promoter not being responsive to the EHV-1 gene 12 also present at low levels in the cell but apparently being responsive to virus infection. Hence, cell lines were produced in which ICP4 under ICP27 promoter and poly(A) control in a plasmid encoding phleomycin resistance was transfected into cells which already effectively allowed the propagation of viruses which lacked ICP27 and were deficient in vmw65 (cell line B5 described above). Phleomycin- and neomycin-resistant colonies were picked and cloned out. However, these were generally found to give only very poor growth of HSV mutants deficient in vmw65, ICP27, and ICP4 (see Table 1), with only 5 of the 140 colonies picked giving significant growth, and even this growth was limited (Fig. 8 shows virus growth on the best of these cell lines, called 27/12/27:4 cells).

After obtaining these disappointing results, we decided to test other promoters driving ICP4 since the ICP27 promoter, for unexplained reasons, provided inappropriate regulation of ICP4. Thus, further phleomycin- and neomycin-resistant cell lines were produced in which ICP4 was driven either by the ICP4 promoter and poly(A) or by the dexamethasone-inducible MMTV promoter and a simian virus 40 poly(A). It was hoped that either correct regulation of ICP4 expression by the ICP4 promoter or dexamethasone-inducible ICP4 expression might provide cell lines capable of improved growth of viruses deficient in vmw65, ICP27, and ICP4. The MMTV promoter was used, as well as the ICP4 promoter, in these experiments, as the low levels of EHV-1 gene 12 already expressed in the cells might be expected to stimulate the ICP4 promoter (see earlier), generating toxic levels of ICP4. It was hoped that this would not be the case if the MMTV promoter was used.

We picked 138 and 88 clones by using the ICP4 and MMTV

27



27/12

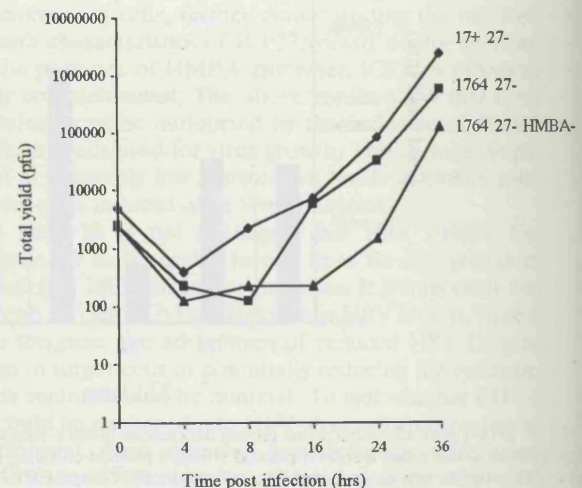


FIG. 5. Growth of HSV mutants on EHV-1 gene 12- and ICP27 gene-containing cell lines. Growth curves of the indicated viruses on cell lines containing either the gene for ICP27 or the gene for ICP27 and EHV-1 gene 12 are shown.

promoters, respectively, and analyzed the virus growth characteristics (Table 1). Of the ICP4 promoter-driven clones, the majority were of only limited permissivity for vmw65/ICP27/ICP4-deficient viruses, although two clones were capable of efficient growth. One of these was selected for further study (27/12/4:4 cells). It was thought that this variability probably reflected positional effects altering the regulation of the ICP4 promoter in the context of EHV gene 12-expressing cells, in some rare cases allowing efficient growth of vmw65/ICP27/ICP4-deficient viruses. However, of the 88 clones picked in which ICP4 expression was controlled by the MMTV promoter, 60 grew ICP4-deficient viruses efficiently (initially with the inclusion of dexamethasone in the medium at the time of inoculation—see below), at least as well as on the two ICP4 promoter-containing cell lines described above. This indicated



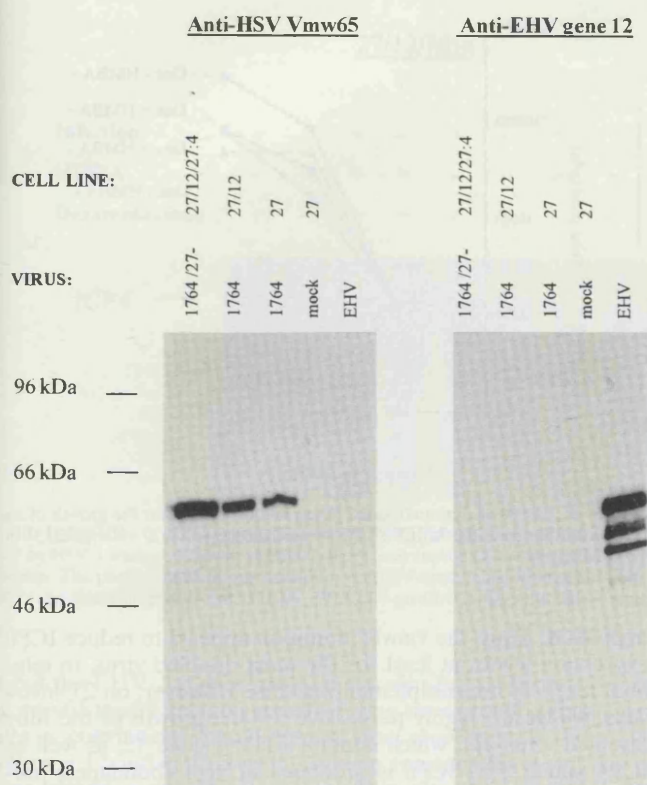


FIG. 6. The EHV-1 gene 12 protein is not packaged into HSV virions. Western blots resulting from polyacrylamide gel electrophoresis of partially purified preparations of each of the viruses indicated grown on the cell lines indicated and probed with either an anti-HSV-1 vmw65 or an anti-EHV-1 gene 12 antibody are shown. Purified EHV-1 was used as a positive control. Viruses were partially purified by freeze-thaw treatment, followed by low-speed centrifugation to remove cell debris and high-speed centrifugation of the resulting supernatants. Electrophoresis was performed on these resuspended high-speed pellets at  $\approx 10^6$  PFU of virus per lane.

that with the MMTV promoter, positional effects are of minimal importance for effective ICP4 regulation in the context of EHV gene 12-containing cell lines, unlike when the ICP4 promoter is used. Again, one clone was selected for further work (27/12/M:4 cells). Figure 8 also shows one-step growth curves resulting from the growth of vmw65/ICP27/ICP4-deficient viruses and vmw65/ICP27-deficient viruses on the best of each of these types of cell line at an MOI of 0.01 (i.e., also on 27/12/4:4 and 27/12/M:4 cells). At higher, more optimal MOIs,  $10^6$  to  $10^7$  PFU/ml can usually be harvested from the culture medium by using the cell lines shown, in which ICP4 expression is driven by either the ICP4 or the MMTV promoter.

Interestingly, inclusion of dexamethasone in the medium at the time of inoculation using cells containing ICP4 under MMTV promoter control did not increase the yield of vmw65/ICP27/ICP4-deficient viruses, suggesting that the MMTV promoter, like the ICP27 promoter, is also responsive to virus infection, but here in a fashion appropriate to allow effective virus growth. The greatest yield of vmw65/ICP27/ICP4-deficient virus was obtained in the presence of HMBA but without dexamethasone (Fig. 9). To confirm and examine the responsiveness of ICP4 expression levels to virus infection, HMBA, and dexamethasone (for 27/12/M:4 cells), Western blotting was performed with each of the cell lines with and without virus infection and with and without HMBA and dexamethasone (for 27/12/M:4 cells; Fig. 10). This showed only very low levels

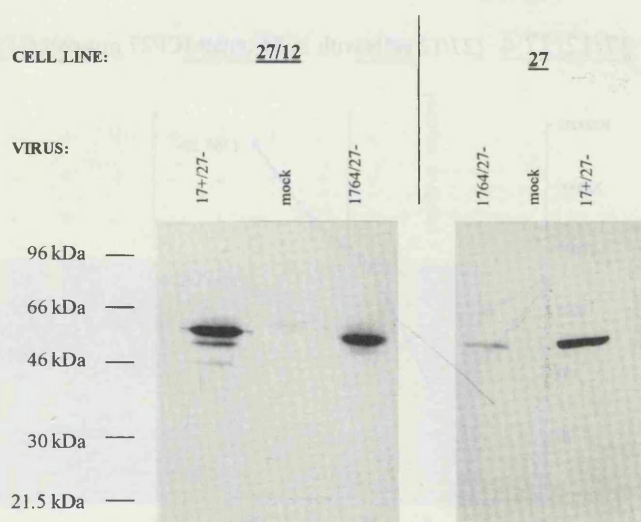


FIG. 7. Virus infection induces expression from the ICP27 promoter in ICP27-containing cells. Shown is a Western blot in which extracts 24 h postinfection of either ICP27-containing or ICP27- and EHV-1 gene 12-containing cells, either mock infected or infected with the indicated viruses (MOI = 5), were probed with an anti-ICP27 antibody.

of ICP4 in 27/12/27:4 cells (in which vmw65/ICP27/ICP4-deficient viruses grow poorly) and higher virus- and HMBA-inducible levels of ICP4 in 27/12/4:4 and 27/12/M:4 cells. Thus, in both of these cell lines, ICP4 levels are constitutively relatively low but expression is stimulated by virus infection and/or HMBA. Dexamethasone also induced ICP4 in 27/12/M:4 cells, but as discussed above, this did not further enhance virus growth. Thus, in the context of EHV-1 gene 12-containing cells, the MMTV promoter appears to be generally induced by virus infection in a position-independent manner (many clones capable of producing virus growth were obtained) and this induction provides optimal ICP4 regulation for virus growth. The ICP4 promoter, on the other hand, can provide virus-inducible expression appropriate for virus growth, but this is probably in a considerably more position-dependent fashion, as such clones effective at producing virus growth were only rarely obtained.

Western blot assays were also performed on BHK and 27/12/M:4 cells infected with either the ICP27-deficient virus, the ICP27/ICP34.5/vmw65-deficient virus, or the ICP27/ICP4/ICP34.5/vmw65-deficient virus and probed with an anti-ICP0 antibody (Fig. 11). This showed that while the ICP27-deficient virus gave significant levels of ICP0 expression in noncomplementing (BHK) cells, the virus deficient in ICP27, ICP34.5, and vmw65 gave only low-level ICP0 expression under such circumstances, while the virus with ICP4 also deleted gave detectable ICP0 expression only after long exposure of Western blots (and thus, ICP0 is not visible in Fig. 11), even at a

TABLE 1. Percentages of BHK clones capable of complementing ICP4 deficiencies in ICP27/ICP4/vmw65-deficient viruses when ICP4 was driven by various promoters

Promoter driving ICP4	No. of clones picked	No. giving significant growth	% Giving significant growth
ICP27	140	5	3.5
ICP4	138	2	1.4
MMTV	88	60	68

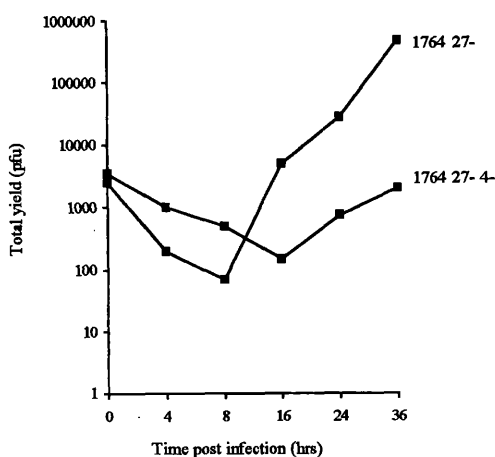
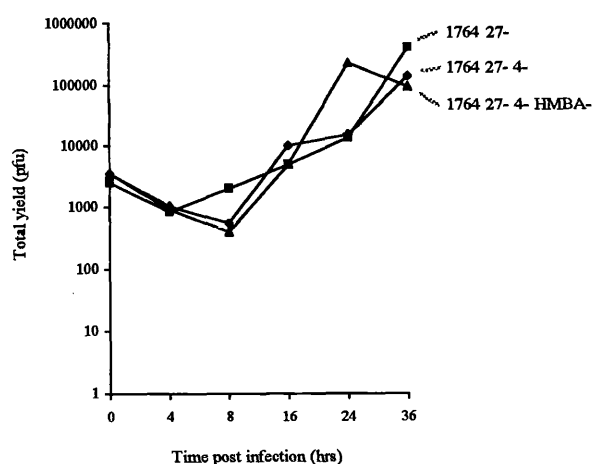
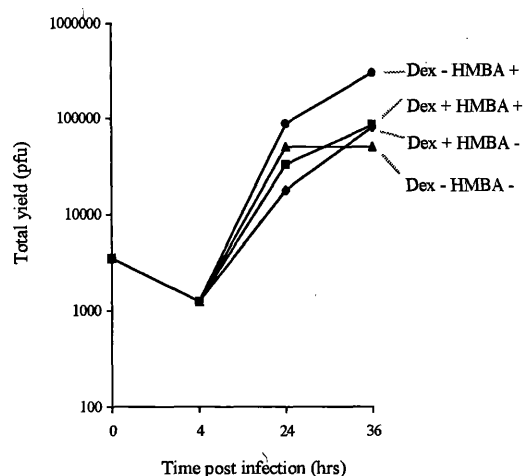
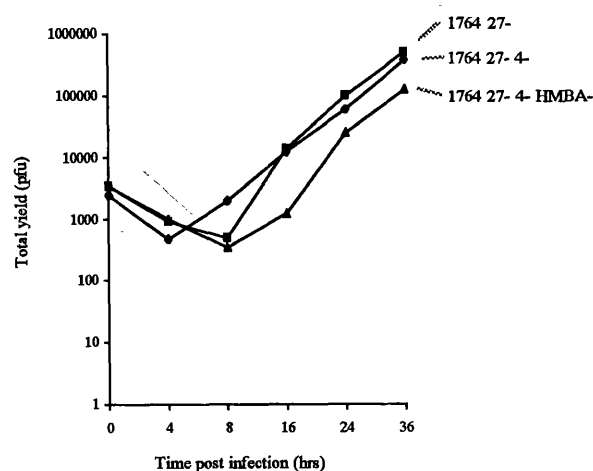
27/12/27:4 (27/12 cells with ICP4 under ICP27 promoter)27/12/4:4 (27/12 cells with ICP4 under ICP4 promoter)27/12/M:4 (27/12 cells with ICP4 under MMTV promoter)

FIG. 9. Effects of dexamethasone (Dex) and/or HMBA on the growth of an HSV-1 mutant deficient in ICP27, ICP4, and *vmw65* (1764/27-4-/pR20.5) in 27/12/M:4 cells.

high MOI. Thus, the *vmw65* mutation appears to reduce ICP0 expression levels, at least for the most disabled virus, to minimal levels in noncomplementing cells. However, on 27/4/M:4 cells, which are highly permissive for the growth of the fully disabled virus and which contain EHV-1 gene 12, as well as ICP4 and ICP27, ICP0 is produced in high abundance, presumably following transactivation of the ICP0 promoter by EHV-1 gene 12.

Thus, taking Fig. 7, 10, and 11 together, a model of IE gene regulation following virus infection of 27/12/M:4 cells would envisage that in the absence of virus infection, EHV-1 gene 12 is expressed weakly from the CMV promoter and that only low levels of ICP27 and ICP4 are produced in the cell. Following infection with an ICP4/ICP27/*vmw65* mutant virus, EHV-1 gene 12 transactivates ICP0 expression from the virus which, in turn, activates further EHV-1 gene 12 expression and the ICP27 and MMTV promoters.

## DISCUSSION

Herpesviruses other than HSV have been shown in a number of studies to encode homologues of *vmw65* (e.g., in EHV-1, varicella-zoster virus, and bovine herpesvirus 1) (3, 8, 29) which, as in HSV, are virion proteins that, in complex with host factors, transactivate viral IE gene promoters soon after virus infection. It has also been shown that for EHV-1 and -4 at least, such proteins can effectively transactivate IE gene promoters from heterologous herpesviruses even though consensus binding sites (TAATGARAT-like motifs) are somewhat different between the different viruses and the level of sequence similarity between the proteins from the different viruses is not particularly high (3, 12, 23). These were thus somewhat surprising findings at the time.

We have extended this work and found that in the case of EHV-1 gene 12, not only can the transactivating activity on HSV IE promoters be demonstrated in cotransfection experi-

FIG. 8. Growth of HSV mutants on cell lines containing EHV-1 gene 12, ICP27, and ICP4. Shown are growth curves of the indicated viruses grown on cell lines containing ICP27 and EHV-1 gene 12 with ICP4 under either ICP27, ICP4, or MMTV promoter control. HMBA was included in the medium during virus growth, except where indicated otherwise.



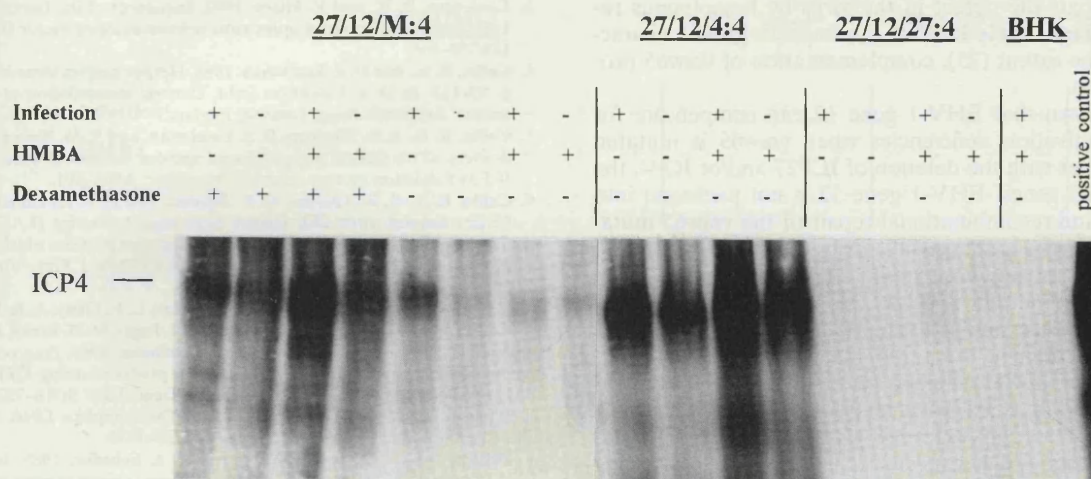


FIG. 10. Inducibility of ICP4 expression levels by virus infection in ICP4-containing cells. Shown are Western blots probed with an anti-ICP4 antibody following growth of an HSV-1 mutant deficient in ICP27, ICP4, and *vmw65* (1764/27-/4-/pR20.5) in the cells indicated and under the conditions indicated at an MOI of 5, 48 h postinfection. The positive control was growth of an ICP4-containing virus strain (1764/27-/pR20) on BHK cells. After long exposure, low-level ICP4 could also be detected in the infection-positive or HMBA 27/12/27:4-positive lanes, although it is not evident in this exposure.

ents but the EHV-1 gene 12 protein can also fully substitute for the *vmw65* transactivating activity during virus growth. This provides a convincing demonstration that even though the EHV-1 gene 12 and *vmw65* proteins have considerably divergent amino acid sequences, they can still perform a fully homologous transactivating function in the virus life cycle which extends to the ability to substitute for the heterologous protein in virus growth. Thus, even though the protein and consensus binding sequences have diverged considerably during evolution, the necessity to retain the ability to interact with very similar host factors in each case has probably led to the generation of proteins that are very different yet can still functionally substitute for one another in virus growth. As well as the lack of sequence similarity between the proteins (34% identical

at the amino acid level, 46% identical at the DNA level; University of Wisconsin Genetics Computer Group GAP program), the large differences between the proteins is further demonstrated by the fact that they differ completely in functional layout (17). Thus, while the transactivating and DNA binding activities of *vmw65* can be localized to specific C- and N-terminal domains, respectively (33, 41), such activities cannot be separated in EHV-1 gene 12, where the whole protein is required in each case (17).

However, while we have found that EHV-1 gene 12 can substitute for transactivation by *vmw65* during the growth of HSV, gene 12 does not appear to be packaged into HSV particles and thus cannot perform the essential structural role of *vmw65* in HSV. This result also confirms that growth deficiencies in HSV-1 containing the *in1814* mutation are indeed due to the deficiency in transactivation associated with the *in1814* mutation rather than due to an effect on the structural function of *vmw65*, which was otherwise formally possible (30). Thus, if a structural effect were the case, growth deficiencies associated with the *in1814* mutation could not be overcome by EHV-1 gene 12, as it is not packaged and thus cannot structurally substitute for *vmw65*.

All of the above information, while of interest in the functional comparison of herpesvirus proteins, also provides a novel means by which the propagation of HSV vector viruses can be improved. Thus, for use as a vector, for which HSV has a number of potential advantages, particularly in the nervous system, the virus has to be disabled so that it is no longer pathogenic and, moreover, is minimally cytotoxic. A number of studies have shown that minimal cytotoxicity can probably only be obtained when IE gene expression is minimized (20–22, 35, 43), preventing toxicity from these highly cytotoxic proteins themselves, and also preventing the expression of most of the  $\approx 80$  other genes in the HSV genome. Mutations of *vmw65* can greatly reduce IE gene expression (1, 39), and these mutations may be particularly attractive in vectors when one or more essential IE genes have also been deleted (see introduction). However, such viruses are highly compromised for growth, even when the essential IE gene defect is otherwise effectively complemented. Moreover, *vmw65* cannot be expressed from the cell line for virus growth, as this would (i) be packaged and

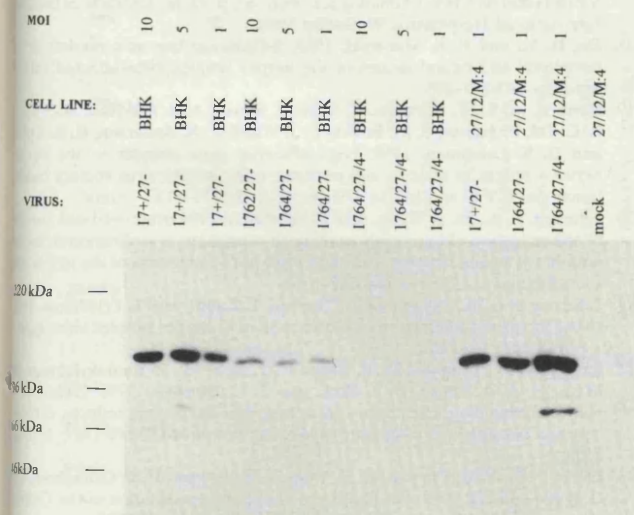


FIG. 11. Induction of ICP0 expression from *vmw65*-deficient viruses during growth on MMTV:4 cells. Shown is a Western blot of 27/12/M:4 cell extracts (24 h postinfection) probed with an anti-ICP0 antibody following growth of the indicated viruses at an MOI of 1 in comparison to extracts of BHK cells (in which virus growth is not possible) inoculated with the various viruses at a range of MOIs. ICP0 is not detectable in BHK cells inoculated with the 1764/27-/4- virus but is abundantly expressed in 27/12/M:4 cells.



(ii) quickly repair the defect in the virus by homologous recombination. Thus, while HMBA can improve growth characteristics to some extent (25), complementation of vmw65 provides a problem.

We have shown that EHV-1 gene 12 can compensate for vmw65 transactivation deficiencies when vmw65 is mutated alone or together with the deletion of ICP27 and/or ICP4, the two essential IE genes. EHV-1 gene 12 is not packaged into HSV virions, and recombinational repair of the vmw65 mutation in vector viruses is not possible by homologous means due to the only minimal sequence similarity between EHV-1 gene 12 and vmw65 generally and as EHV-1 gene 12 does not encode any sequence similar to the region altered in vmw65.

We have also defined the parameters necessary for the efficient production of EHV-1 gene 12-containing cell lines containing ICP4 and/or ICP27 such that these proteins are only significantly expressed in response to virus infection. ICP4 and ICP27 would otherwise be expected to be cytotoxic, preventing the generation of such cell lines in a stable fashion. Here the virus-cell line combinations used have only minimal DNA sequence overlap ( $\approx 30$  bp at one end of ICP4), and thus, recombinational repair of any of the deficiencies in the vector viruses is again minimized. Efficient growth of HSV with essential IE gene deletions and incorporation of vmw65 transactivation deficiencies was not previously possible and potentially provides advantages over deletion or inactivation of ICP27, ICP4, ICP0, and ICP22 individually. These are the four regulatory IE genes, and for optimally efficient growth of such disabled viruses, cell lines containing all of the genes would need to be produced, although ICP0 and ICP22 are not absolutely essential for virus growth. As each of these four IE genes is highly cytotoxic, such cell lines are hard to generate, and while cells containing ICP27, ICP4, and ICP0 have been produced (36), no cell line containing all four of these IE genes has been reported.

The virus-cell line combinations reported here may thus provide an alternative to the deletion or inactivation of all of the IE genes for the production of nontoxic HSV vectors and the concurrent problem of the generation of cell lines allowing their efficient growth. Other non-HSV vmw65 homologues may also be used in a similar way. Particularly when combined with deletions in other nonessential genes which are either virion proteins or would be expected to be expressed in the absence of IE gene expression (e.g., the virion host shutoff protein [vhs], ICP34.5, ORF1, or ICP6), such viruses might be anticipated to be minimally toxic and yet still be capable of efficient growth for vector stock production *in vitro*.

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Suzanne Thomas and Caroline Lilley contributed equally to this work.

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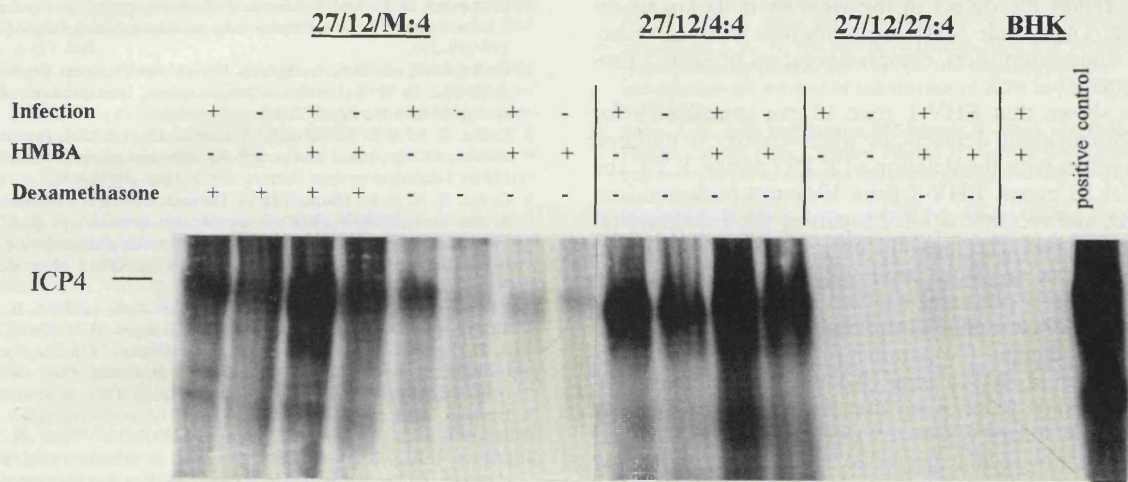


FIG. 10. Inducibility of ICP4 expression levels by virus infection in ICP4-containing cells. Shown are Western blots probed with an anti-ICP4 antibody following growth of an HSV-1 mutant deficient in ICP27, ICP4, and *vmw65* (1764/27-/4-/pR20.5) in the cells indicated and under the conditions indicated at an MOI of 5, 48 h postinfection. The positive control was growth of an ICP4-containing virus strain (1764/27-/pR20) on BHK cells. After long exposure, low-level ICP4 could also be detected in the infection-positive or HMBA 27/12/27:4-positive lanes, although it is not evident in this exposure.

ments but the EHV-1 gene 12 protein can also fully substitute for the *vmw65* transactivating activity during virus growth. This provides a convincing demonstration that even though the EHV-1 gene 12 and *vmw65* proteins have considerably divergent amino acid sequences, they can still perform a fully homologous transactivating function in the virus life cycle which extends to the ability to substitute for the heterologous protein in virus growth. Thus, even though the protein and consensus binding sequences have diverged considerably during evolution, the necessity to retain the ability to interact with very similar host factors in each case has probably led to the generation of proteins that are very different yet can still functionally substitute for one another in virus growth. As well as the lack of sequence similarity between the proteins (34% identical

at the amino acid level, 46% identical at the DNA level; University of Wisconsin Genetics Computer Group GAP program), the large differences between the proteins is further demonstrated by the fact that they differ completely in functional layout (17). Thus, while the transactivating and DNA binding activities of *vmw65* can be localized to specific C- and N-terminal domains, respectively (33, 41), such activities cannot be separated in EHV-1 gene 12, where the whole protein is required in each case (17).

However, while we have found that EHV-1 gene 12 can substitute for transactivation by *vmw65* during the growth of HSV, gene 12 does not appear to be packaged into HSV particles and thus cannot perform the essential structural role of *vmw65* in HSV. This result also confirms that growth deficiencies in HSV-1 containing the *in1814* mutation are indeed due to the deficiency in transactivation associated with the *in1814* mutation rather than due to an effect on the structural function of *vmw65*, which was otherwise formally possible (30). Thus, if a structural effect were the case, growth deficiencies associated with the *in1814* mutation could not be overcome by EHV-1 gene 12, as it is not packaged and thus cannot structurally substitute for *vmw65*.

All of the above information, while of interest in the functional comparison of herpesvirus proteins, also provides a novel means by which the propagation of HSV vector viruses can be improved. Thus, for use as a vector, for which HSV has a number of potential advantages, particularly in the nervous system, the virus has to be disabled so that it is no longer pathogenic and, moreover, is minimally cytotoxic. A number of studies have shown that minimal cytotoxicity can probably only be obtained when IE gene expression is minimized (20–22, 35, 43), preventing toxicity from these highly cytotoxic proteins themselves, and also preventing the expression of most of the ≈80 other genes in the HSV genome. Mutations of *vmw65* can greatly reduce IE gene expression (1, 39), and these mutations may be particularly attractive in vectors when one or more essential IE genes have also been deleted (see introduction). However, such viruses are highly compromised for growth, even when the essential IE gene defect is otherwise effectively complemented. Moreover, *vmw65* cannot be expressed from the cell line for virus growth, as this would (i) be packaged and

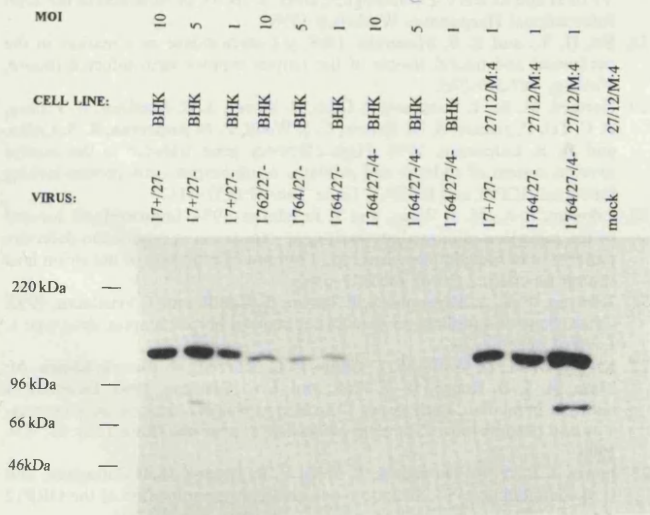


FIG. 11. Induction of ICP0 expression from *vmw65*-deficient viruses during growth on MMTV:4 cells. Shown is a Western blot of 27/12/M:4 cell extracts (24 h postinfection) probed with an anti-ICP0 antibody following growth of the indicated viruses at an MOI of 1 in comparison to extracts of BHK cells (in which virus growth is not possible) inoculated with the various viruses at a range of MOIs. ICP0 is not detectable in BHK cells inoculated with the 1764/27-/4- virus but is abundantly expressed in 27/12/M:4 cells.

(ii) quickly repair the defect in the virus by homologous recombination. Thus, while HMBA can improve growth characteristics to some extent (25), complementation of *vmw65* provides a problem.

We have shown that EHV-1 gene 12 can compensate for *vmw65* transactivation deficiencies when *vmw65* is mutated alone or together with the deletion of ICP27 and/or ICP4, the two essential IE genes. EHV-1 gene 12 is not packaged into HSV virions, and recombinational repair of the *vmw65* mutation in vector viruses is not possible by homologous means due to the only minimal sequence similarity between EHV-1 gene 12 and *vmw65* generally and as EHV-1 gene 12 does not encode any sequence similar to the region altered in *vmw65*.

We have also defined the parameters necessary for the efficient production of EHV-1 gene 12-containing cell lines containing ICP4 and/or ICP27 such that these proteins are only significantly expressed in response to virus infection. ICP4 and ICP27 would otherwise be expected to be cytotoxic, preventing the generation of such cell lines in a stable fashion. Here the virus-cell line combinations used have only minimal DNA sequence overlap ( $\approx 30$  bp at one end of ICP4), and thus, recombinational repair of any of the deficiencies in the vector viruses is again minimized. Efficient growth of HSV with essential IE gene deletions and incorporation of *vmw65* transactivation deficiencies was not previously possible and potentially provides advantages over deletion or inactivation of ICP27, ICP4, ICP0, and ICP22 individually. These are the four regulatory IE genes, and for optimally efficient growth of such disabled viruses, cell lines containing all of the genes would need to be produced, although ICP0 and ICP22 are not absolutely essential for virus growth. As each of these four IE genes is highly cytotoxic, such cell lines are hard to generate, and while cells containing ICP27, ICP4, and ICP0 have been produced (36), no cell line containing all four of these IE genes has been reported.

The virus-cell line combinations reported here may thus provide an alternative to the deletion or inactivation of all of the IE genes for the production of nontoxic HSV vectors and the concurrent problem of the generation of cell lines allowing their efficient growth. Other non-HSV *vmw65* homologues may also be used in a similar way. Particularly when combined with deletions in other nonessential genes which are either virion proteins or would be expected to be expressed in the absence of IE gene expression (e.g., the virion host shutoff protein [vhs], ICP34.5, ORFP, or ICP6), such viruses might be anticipated to be minimally toxic and yet still be capable of efficient growth for vector stock production *in vitro*.

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## BRIEF COMMUNICATION

# Gene transfer using a disabled herpes virus vector containing the EMCV IRES allows multiple gene expression *in vitro* and *in vivo*

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*The design of recombinant HSV-1 vectors for delivery of transgenes to the central nervous system is undergoing constant development. Problems associated with the construction and use of such vectors include the requirement for detection of recombinant versus nonrecombinant virus in vitro and also the identification of transduced cells in vivo. This could be overcome by the insertion of reporter genes such as lacZ or green fluorescent protein (GFP) under a separate promoter to the transgene to be*

*expressed. In this case, however, reporter gene expression does not necessarily confirm transgene expression as a separate RNA must be produced. This study reports the use of an encephalomyocarditis virus internal ribosome entry site (IRES) to enable the translation of two reporter genes from a single mRNA transcript driven by the same promoter within a disabled HSV vector, and discusses the potential advantages of this approach.*

**Keywords:** IRES; HSV-1; gene delivery; bicistronic vectors

We and others have successfully used recombinant herpes simplex virus type 1 (HSV-1) vectors to deliver reporter genes to the peripheral and central nervous system.<sup>1-3</sup> As more efficient vectors are being designed, the potential of gene delivery for the treatment of disease is becoming a real possibility. However, the expression of more than one gene may be required for such treatments to be of maximum benefit, or alternatively, the expression of a reporter gene in addition to the functional transgene could aid experimental assessment of gene transfer efficiencies and optimisation of the gene transfer process.

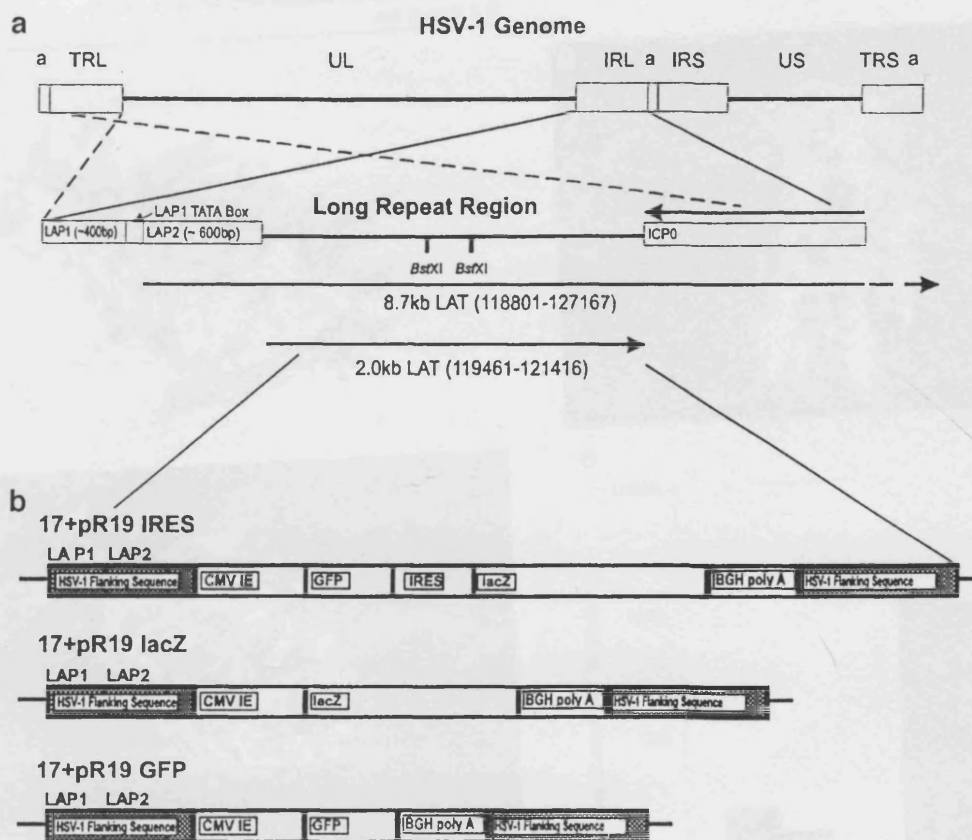
The current strategies for expressing two transgenes in the same gene delivery vector can be either to use two promoters, thus expressing each gene separately, or to insert an internal ribosome entry site (IRES), from encephalomyocarditis virus (ECMV) or another picornavirus, between the two transgenes to enable transcription of both coding regions from a single promoter and thus translation of both genes from a single mRNA.<sup>4</sup> The potential problem with the use of two promoters in a viral vector is that the promoter driving reporter gene activity may not mimic the activity of the promoter driving the functional transgene. If an identical promoter is used to drive the expression of each transgene separately, the two promoters may be susceptible to homologous recombination resulting in an unstable vector virus or competition for endogenous factors may reduce gene expression levels. Also, as recombinant virus plaques are

often selected by reporter gene activity, false positives may be selected during plaque purification.

The EMCV IRES enables cap-independent translation of mRNA. In a defective HSV-1 amplicon vector it was demonstrated that insertion of the EMCV IRES between the glucose transporter gene (upstream) and the reporter gene *lacZ*, encoding  $\beta$ -galactosidase, (downstream) successfully enabled translation of both genes from a single mRNA species when transcribed under the control of the HSV-1 ICP4 promoter.<sup>5,6</sup> It was also reported that gene expression by cap-independent translation by way of the IRES was not as efficient as cap-dependent translation. The EMCV IRES has also been used successfully to generate bicistronic transcripts in adenoviral, adeno-associated viral and retroviral gene delivery strategies.<sup>7-9</sup>

The use of recombinant HSV-1 vectors for transgene delivery to the central nervous system has several potential advantages over other viral strategies. Recombinant HSV-1 vectors that have had one or more essential genes deleted can enter a latent state in neurons, transcribing only the latency-associated transcript (LAT) and thus they provide the possibility of long-term gene transfer to the nervous system. HSV also has a large genome (152 kb), and thus large sequences can be stably inserted into the locus of choice, which is not always the case with other vectors where packaging capabilities are more limited. Unlike HSV amplicon vectors which are grown with a helper virus, often resulting in unreliable ratios of amplicon and helper virus in such stocks, the growth of disabled HSV vectors is less problematical as long as a cell line effectively complementing the deletions in the virus is used.

We aimed to use a similar approach, using an ECMV



**Figure 1** Schematic representation of the 17+pr19-based HSV-1 vectors. The long repeat region of the HSV-1 genome is shown (not to scale). In (a) the loci of the LAT promoters, the coding regions of the major and minor LATs and ICP0 (IE1) are shown. The structure of the expression cassettes inserted into the LAT region is shown in (b). The CMV IE promoter and bovine growth hormone polyadenylation sequence (BGH poly A) were excised from pcDNA3 (Invitrogen, De Schelp, The Netherlands) using *Nru*I and *Bbs*I and inserted between the genomic *Bst*XI sites of a 3.5 kb *Not*I fragment derived from the long repeat regions of the HSV-1 genome (base pairs 118439–122025). The *lacZ* gene was excised from pCH110 (Pharmacia, Uppsala, Sweden) using *Hind*III and *Bam*HI, and the GFP gene excised from pEGFPN1 (Clontech, Palo Alto, CA, USA) using *Hind*III and *Not*I and inserted between similar sites downstream of the CMV IE promoters now in the LAT region to make 17+pr19 *lacZ* and 17+pr19 GFP, respectively. The EMCV IRES<sup>4</sup> was excised from pCITE-1 using *Eco*RI and *Nco*I and inserted upstream of the *lacZ* gene in frame with the coding region. The IRES/*lacZ* was excised and inserted into the *Xba*I site downstream of the GFP gene in 17+pr19 GFP to make 17+pr19 IRES. Recombinant viruses were generated by homologous recombination using a method adapted from that described by Stow and Wilkie.<sup>1,12</sup>

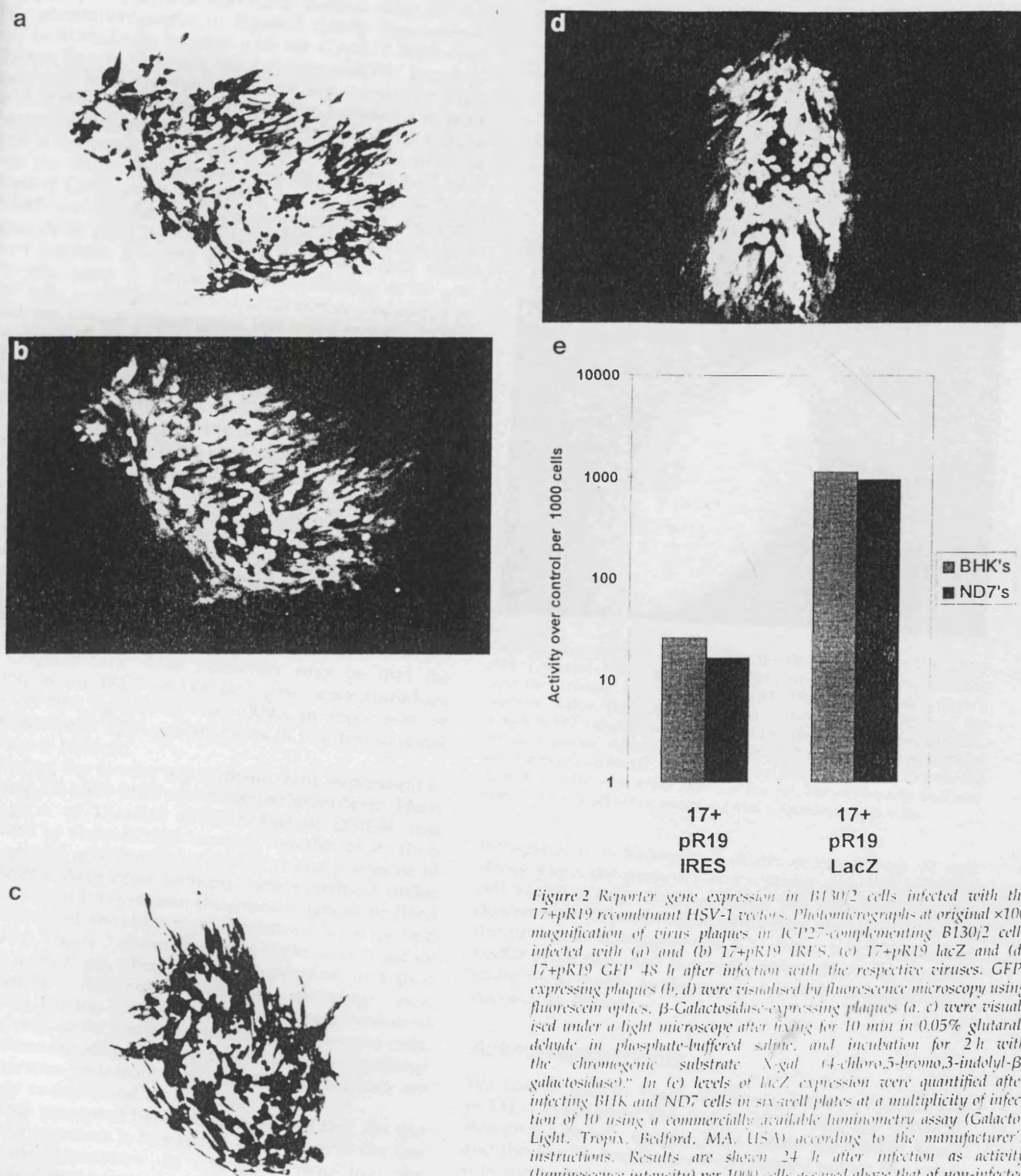
IRES in the context of a recombinant disabled HSV-1 vector, to that which has previously been used in an amplicon vector and reported previously.<sup>5,6</sup> We therefore inserted an EMCV IRES between the green fluorescent protein (GFP) and *lacZ* genes driven by a CMV promoter in a virus deleted for the essential gene ICP27, and compared the expression of the two reporter genes with similar recombinant viruses in which only a single reporter gene is inserted.

The IRES has previously been used in a recombinant HSV-1 backbone, downstream of the LAT promoter,<sup>3</sup> but only to express a single gene. Here, *lacZ* was inserted in place of the 2 kb LAT downstream of the LAT promoter. There was a 1.5 kb sequence between the LAT promoter and the *lacZ* gene which contains 10 AUG codons thereby preventing ribosomal access to the *lacZ* start codon. Insertion of the IRES directly upstream to the *lacZ* gene allowed cap-independent translation of the reporter gene irrespective of the start site of transcription and stable expression of the *lacZ* gene during latency was achieved.

In order to extend these data to allow the expression of pairs of genes, we report *in vitro* that not only are both

genes effectively expressed, albeit at lower levels than with either virus expressing the genes individually, but that both genes are also effectively expressed in the rat brain *in vivo*.

We have previously shown that recombinant HSV-1 vectors disabled by deletion of the infected cell protein 27 (ICP27) essential gene successfully infect and express transgenes in both cardiac and neuronal cells *in vivo*.<sup>10,11</sup> Therefore, the vectors used for this study were also deleted for ICP27. Transgene expression was driven by the human cytomegalovirus immediate-early (CMV IE) promoter and the promoter/transgene cassette was inserted by homologous recombination into the 2 kb LAT sequence in the HSV-1 genome (strain 17+). We have found that a CMV promoter placed at this position in viruses which are attenuated but still replication competent allows efficient gene expression during latency in dorsal root ganglia (DRGs) after footpad inoculation of mice (unpublished results), but in the brain or in DRGs using a virus deleted for only ICP27, long-term expression is only seen in a few cells. This is probably due to remaining toxicity of ICP27-deleted viruses in the



**Figure 2** Reporter gene expression in B130/2 cells infected with the 17+pR19 recombinant HSV-1 vectors. Photomicrographs at original  $\times 100$  magnification of virus plaques in ICP27-complementing B130/2 cells infected with (a) and (b) 17+pR19 IRES, (c) 17+pR19 LacZ and (d) 17+pR19 GFP 48 h after infection with the respective viruses. GFP-expressing plaques (b, d) were visualised by fluorescence microscopy using fluorescein optics.  $\beta$ -Galactosidase-expressing plaques (a, c) were visualised under a light microscope after fixing for 10 min in 0.05% glutaraldehyde in phosphate-buffered saline, and incubation for 2 h with the chromogenic substrate X-gal (4-chloro,5-bromo,3-indolyl- $\beta$ -galactosidase). In (e) levels of LacZ expression were quantified after infecting BHK and ND7 cells in six-well plates at a multiplicity of infection of 10 using a commercially available luminometry assay (Galacto-Light, Tropix, Bedford, MA, USA) according to the manufacturer's instructions. Results are shown 24 h after infection as activity (luminescence intensity) per 1000 cells assayed above that of non-infected control cells.

brain, and due to a requirement for limited replication for efficient delivery to DRGs after footpad inoculation. The ICP27-deleted viruses reported here are thus only appropriate for testing the activity of promoters in the short term in the brain, and thus the effectiveness of the HCMV IRES at allowing the expression of pairs of genes

in the context of the HSV genome was only tested at such times in the experiments reported here.

Three recombinant viruses were constructed: 17+pR19 LacZ, 17+pR19 GFP and 17+pR19 IRES, containing either GFP, LacZ, or GFP and LacZ separated by the IRES, respectively. These are shown in Figure 1. Each virus was



plaque purified following homologous recombination of the respective plasmids into ICP27-deleted virus DNA.<sup>11</sup> The photomicrographs in Figure 2 clearly demonstrate that in B130/2 cells infected with the 17+pR19 IRES virus (Figure 2a and b) where the deletion of ICP27 is complemented, both reporter genes are expressed to a high level. However, it is also clear that the expression of both the transgene upstream and downstream of the EMCV IRES is decreased in comparison to B130/2 cells infected with the single transgene-expressing 17+pR19 *lacZ* and 17+pR19 GFP viruses (Figure 2c and d). When the level of *lacZ* activity (ie the gene downstream of the IRES sequence in the 17+pR19 IRES virus) was directly compared between 17+pR19 IRES and 17+pR19 *lacZ* when each was used to infect noncomplementing BHK and ND7 cells (a cell line derived from sensory neurons) in which the viruses cannot grow, this result was confirmed showing a 10- to 15-fold reduction in activity when the *lacZ* gene was preceded by the IRES sequence in each case (Figure 2e). The GFP fluorescence intensity was also visibly reduced. Thus, while the expression of pairs of genes can be facilitated by the use of an ECMV IRES in a disabled HSV vector, gene expression levels are reduced compared with the situation where each gene is expressed separately.

It has previously been noted that cap-independent translation is not as effective as cap-dependent translation.<sup>5</sup> This would explain the decreased levels of expression of *lacZ* in cells infected with 17+pR19 IRES compared with similar cells infected with 17+pR19 *lacZ*, but does not explain why the level of expression of the GFP gene is also reduced compared with cells infected with 17+pR19 GFP. One possibility may be that the addition of an IRES or the *lacZ* gene may introduce sequences that destabilise the mRNA in some way or otherwise affects RNA processing such that translational efficiency is reduced.

Following the *in vitro* experiments, gene expression *in vivo* using 17+pR19 IRES was tested in the rat brain. Here,  $2 \times 10^6$  p.f.u. of 17+pR19 IRES in 4  $\mu$ l of DMEM was inoculated by stereotactical injection into the rat striatum under general anaesthetic, uniformly over a period of 10 min. After 2 days brain sections were visualised under UV light for GFP expression (fluorescein optics), or fixed and stained with the chromogenic substrate X-gal for *lacZ* expression. Figure 3 shows the same brain section before staining with X-gal, showing GFP expression, and then subsequently following staining, showing *lacZ* expression. During the staining process co-expression of GFP and *lacZ* could easily be visualised in the same cells, clearly demonstrating that the ECMV IRES is functional in the nervous system *in vivo*, and that both genes are effectively co-expressed.

The work presented here therefore shows that the use of an IRES sequence is a practical alternative to the use of separate promoters in strategies requiring the expression of two transgenes in the context of a recombinant HSV-1 vector. The ECMV IRES allows effective expression of pairs of genes both *in vitro* and in the rat central nervous system *in vivo*. The principle potential advantage here is that if a reporter gene is inserted downstream of the EMCV IRES, and expression is detected, then any upstream gene must also have been transcribed and is thus likely to have been expressed. This may be useful, for example, when quantifying the effects of a

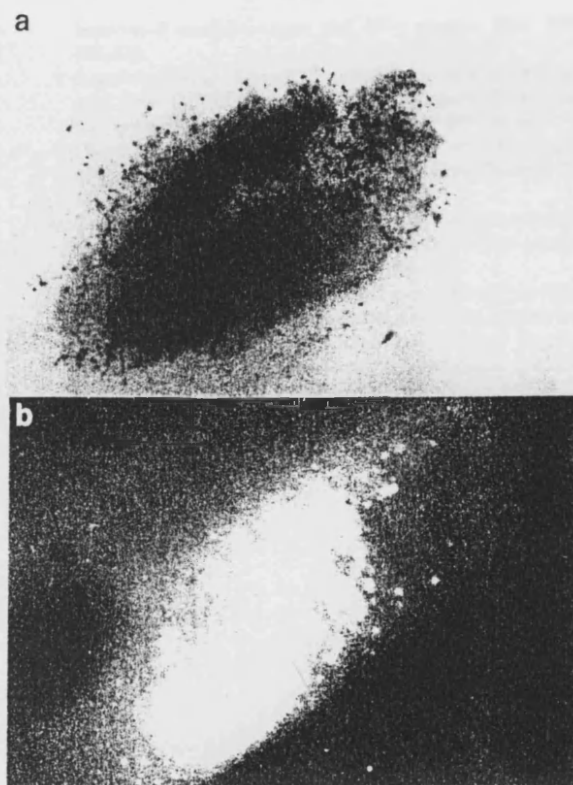


Figure 3 Reporter gene expression in the rat brain *in vivo*. Two 120 g male Lewis rats under general anaesthetic were injected stereotactically into the striatum with 4  $\mu$ l of DMEM (Dulbecco's modified Eagle's medium; Gibco, Bethesda, MA, USA) containing  $2 \times 10^6$  p.f.u. of the 17+pR19 IRES recombinant HSV-1 vector. The injections were continuous over 10 min. After a recovery period of 2 days, the rats were killed and the brains sectioned. The photomicrographs below are taken from the same area of the same brain section of one rat. The section was fixed and stained with X-gal (a) or visualised under fluorescein optics (b).

transgene in reducing cell death in conditions of cell stress. Here, the presence of a reporter gene can confirm cell viability over a time-course, the expression of the downstream reporter also mimicking the expression of the upstream transgene which is under test. Bipromoter vector strategies may not necessarily effectively demonstrate these points as the expression of both genes is not necessarily linked.

### Acknowledgements

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## BRIEF COMMUNICATION

# Pure populations of transduced primary human cells can be produced using GFP expressing herpes virus vectors and flow cytometry

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*Herpes simplex virus (HSV) has often been suggested as a vector for gene delivery to the nervous system although it is also capable of infecting many other cell types. HSV also has the ability to package large genetic insertions so the expression of multiple genes from a single virus is possible. Here we show that a green fluorescent protein (GFP) expressing HSV1 vector can transduce two primary human cell types – quiescent human CD34<sup>+</sup> hematopoietic progenitor cells and dendritic cells – which are both hard to transduce by other means. We also show that GFP is an effective marker when expressed from an HSV vector in vivo in the mouse brain. When GFP is expressed together with a second gene (in this case lacZ) from a single virus, transduced GFP-positive CD34<sup>+</sup> hematopoietic*

*progenitor cells or dendritic cells can both be generated at an effective efficiency of 100% for the second gene. Here transduction with the vector is combined with flow cytometry allowing GFP-positive cells to be sorted from the untransduced population. Such completely transduced populations of quiescent CD34<sup>+</sup> hematopoietic progenitor and dendritic cells cannot easily be achieved by other means, and might thus allow experimental or therapeutic protocols to be carried out requiring high-level transduction which would not otherwise be possible. Such an approach using HSV vectors might also be applicable to other cell types for which transduction is as yet unreliable or of low efficiency.*

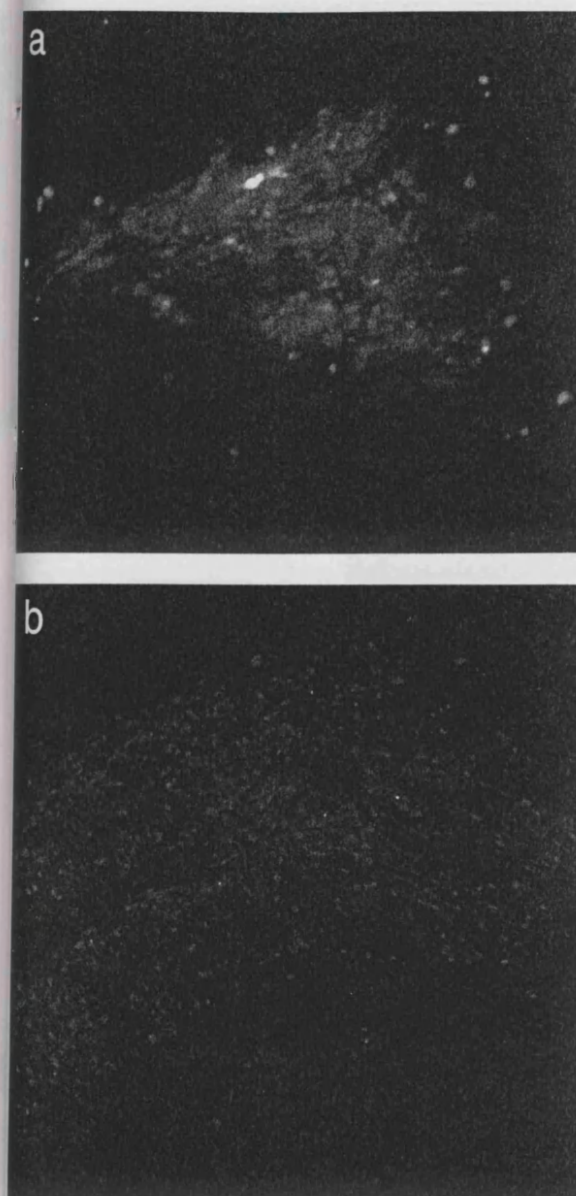
**Keywords:** herpes vector; dendritic cells; hematopoietic progenitor cells

Herpes simplex virus (HSV) vectors have great potential for gene transfer to the nervous system (reviewed in Ref. 1) and also other targets, particularly cell types which are nondividing and/or are hard to transfect by other means. However, without effective disablement such vectors are often toxic to particular cell types, and so combinations of genes must be removed to optimize gene transfer characteristics for a particular target. A second problem with the use of HSV as a vector is that gene expression tends to continue only in the short term after gene transfer, although promoter systems developed by us (unpublished results) and others (see Ref. 1) can now allow expression to continue for at least a number of months. We have previously reported the use of HSV for gene transfer to the central and peripheral nervous systems<sup>2</sup> and the heart<sup>3</sup> and here demonstrate gene transfer to hematopoietic progenitor and dendritic cells which are hard to transduce by other means. Herpes viruses also have the advantage that they can accept large insert sizes,

which could include multiple genes and/or regulatory elements and which is often not possible with other vector systems such as retro-, adeno-, or adeno-associated viruses where the size of insert is limited (for reviews see Ref. 4). The ability to express multiple genes might be important in many therapeutic situations such as, for example, in motor neuron disease where it has recently been shown that the use of two neurotrophic factor expressing adenoviruses was more effective at preventing motor neuron degeneration in an animal model than was either virus alone.<sup>5,6</sup> Similarly multiple gene expression may be useful where a marker for transduced cells is required together with delivery of a 'test' gene in studies of gene function. Alternatively, as we show here, the marker gene can be used for the purification of cells transduced *ex vivo* to homogeneity when combined with flow cytometry.

Previously, recombinant herpes viruses were often made expressing *lacZ* as a marker gene (eg see Ref. 2). However, much interest has recently been shown in green fluorescent protein (GFP) as a marker as it allows expression monitoring in live cells,<sup>7</sup> and GFP expressing retro- and adenovirus vectors have been reported.<sup>8,9</sup> The availability of newer mutants of GFP exhibiting enhanced fluorescence characteristics has considerably improved

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**Figure 1** GFP expression *in vitro* and *in vivo*. (a) BHK cells showing a GFP expressing plaque after infection with an HSV vector with a CMV-GFP insertion into the UL43 gene generated as described previously.<sup>2</sup> Cells were viewed using fluorescence microscopy and fluoroscein optics. (b) A brain section (midbrain) is shown 3 days after intracranial inoculation<sup>2</sup> of a 3-week-old BALB/c mouse using  $1 \times 10^6$  p.f.u. of the GFP expressing HSV vector.

the effectiveness of GFP as a marker gene. We have used the EGFP mutant (Clontech) expressed from a recombinant herpes virus vector and shown that GFP can be used as an effective marker gene within the herpes genome both *in vitro* (Figure 1a) and *in vivo* (Figure 1b). Here we could detect strong fluorescence in brain slices after intracranial inoculation of a GFP expressing HSV vector into the brains of mice. Moreover, as visualisation of GFP can be carried out under physiological conditions, fluorescence can easily be observed in live cells which raises the possibility of either performing physiological

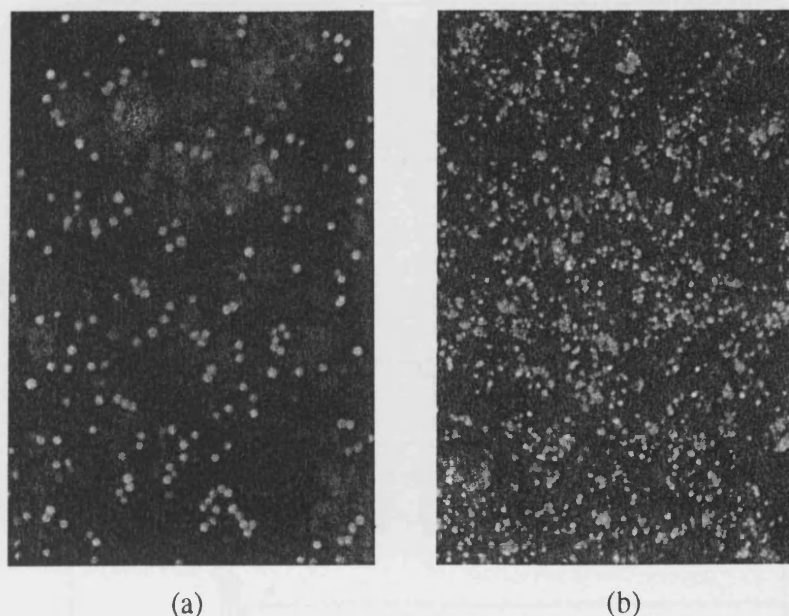
experiments on GFP-positive cells identified as having been transduced *in vivo*, or of sorting transduced cells from the non-transduced background population using a standard flow cytometer. When combined with the ability of the HSV genome to accept inserts of large size this could allow the delivery of GFP together with a second or multiple proteins with subsequent flow cytometric sorting of the GFP-positive cells. Such a procedure would allow an effective transduction efficiency of 100% for the second protein after sorting, and might thus be useful for many experimental or therapeutic situations. We therefore tested the feasibility of this approach using dendritic and hematopoietic progenitor cells as target cell types.

Dendritic cells and CD34<sup>+</sup> hematopoietic progenitor cells are both cell types which are hard to transfect by many routine methods and which could provide important *ex vivo* gene transfer targets for either immunotherapy (eg antigen loading of dendritic cells,<sup>10</sup> or cytokine expression in tumor cells<sup>11</sup>) or corrective gene therapy if effective transduction could be routinely achieved. Although previous experiments using retroviruses and adenoviruses have shown successful transduction of dendritic cells – retroviruses giving approximately 25–65% gene transfer with three rounds of infection over 7 days,<sup>12,13</sup> and adenoviruses giving up to 95% efficiency<sup>14</sup> – dendritic cell transduction is still problematic. Quiescent CD34<sup>+</sup> hematopoietic progenitor cells cannot be transduced by retroviruses or at only low efficiency with adeno-associated virus (eg see Refs 15 and 16), although adenoviruses have been reported to give up to 45% transduction efficiency,<sup>17</sup> and approximately 90% transduction has recently been reported using an HSV2 mutant originally developed as a vaccine.<sup>18</sup> In both these cases CD34<sup>+</sup> cells were derived from bone marrow, whereas the efficiency of transduction using CD34<sup>+</sup> cells mobilized into peripheral blood was approximately 25% using an adenovirus vector.<sup>17</sup> However, in our hands, quiescent CD34<sup>+</sup> progenitor cells cannot be transduced with identical adenovirus vectors other than at low efficiency (<1%, results not shown).

We have now tested an HSV1 vector expressing both GFP (from a CMV promoter) and *lacZ* (from an RSV promoter) for transduction of dendritic cells and CD34<sup>+</sup> hematopoietic progenitor cells, both derived from the peripheral blood of human volunteers/patients. In these experiments we show that HSV1 can be used to transduce these cells at varying efficiency (>70% for dendritic cells and approximately 10% for hematopoietic progenitor cells; Figure 2). Both quiescent (cultured in stem cell factor alone) and cytokine-treated (cultured in stem cell factor, IL3 and IL6) CD34<sup>+</sup> hematopoietic progenitor cells were transduced at equal efficiency in these experiments. Results are thus only shown for quiescent cells. For dendritic cells the transduction efficiency is similar to previous results reported with adenovirus, and shows an improvement for hematopoietic cells which in our hands could not previously be transduced (see above).

Moreover, after cell sorting for GFP-positive cells (Figure 3a), a 100% *lacZ* expressing population could routinely be produced for both dendritic and hematopoietic progenitor cells (Figure 3b). *LacZ* expression levels appeared somewhat variable in dendritic cells, whereas in hematopoietic progenitor cells expression levels were more uniform. As the virus expresses both GFP and *lacZ*,





CD34+ve hematopoietic progenitor cells

Dendritic cells

**Figure 2** Transduction of CD34<sup>+</sup> hematopoietic progenitor cells and dendritic cells using an HSV vector expressing GFP and *lacZ*. CD34<sup>+</sup> hematopoietic progenitor cells were isolated from a patient with Hodgkin's disease mobilized with G-CSF (5.8 µg/kg/day for 10 days) and cyclophosphamide (1.5 g/m<sup>2</sup>). Erythrocytes were removed (Redout, Robbins Scientific, Sunnyvale, CA, USA), mononuclear cells (PBMCs) isolated by density centrifugation (Ficoll-Paque (Pharmacia, Uppsala, Sweden)), washed with PBS, 0.5% BSA, 1 mg/ml EDTA, resuspended in 400 µl CD34 multisort beads (Miltenyi Biotec, Camberley, UK) per 4 × 10<sup>6</sup> cells, and CD34<sup>+</sup> cells isolated (two rounds) using Miltenyi MACS columns (VS+, Miltenyi Biotec) and QBend10, a CD34 class II antibody.<sup>19</sup> Purity was assessed as >98% by APAAP staining of cytospin preparations with a CD34 antibody (HPCA-2 mAb), with an isotype-matched control. Cells were cultured in Iscove's medium-20% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 10 ng/ml stem cell factor (R&D Systems, Abingdon, UK). Cytokine-stimulated CD34<sup>+</sup> cells (not shown) were cultured identically but including 10 ng/ml IL-3 and IL-6 (both Novartis, Sandwich, UK) in the media. Dendritic cells were prepared from Ficoll lymphoprep (Nycomed Pharma, Oslo, Norway) purified PBMCs using 2 h-adherent cells cultured in RPMI, 10% FCS, 50 µM 2-mercaptoethanol containing 100 ng/ml GM-CSF and 50 ng/ml IL-4 (both provided by Schering-Plough, Kenilworth, NJ, USA). After 7 days cultures were depleted of CD19<sup>+</sup> B and CD3<sup>+</sup>, CD2<sup>+</sup> T cells using magnetic beads (Dyna, Oslo, Norway).<sup>20</sup> Cells expressed characteristic dendritic cell markers: high HLA DQ (86%), HLA DR (95%) and CD1a (95%), and low CD14 (22%), and are potent T cell proliferation activators in a mixed leukocyte reaction (data not shown). Hematopoietic or dendritic cells (approximately 1 × 10<sup>6</sup>) were pelleted and resuspended in 100 µl DMEM containing approximately 1 × 10<sup>6</sup> p.f.u. of the HSV vector for 2 h at 37°C and then cultured at 2 × 10<sup>5</sup> cells/ml in the appropriate medium (above) at 37°C/5% CO<sub>2</sub> for 24 h. The HSV vector contained a cassette consisting of a CMV promoter driving GFP (E-GFP; Clontech, Palo Alto, CA, USA) and an RSV promoter driving *lacZ* and inserted into the UL43 gene as reported previously.<sup>2</sup> Tissue culture reagents were purchased from Gibco (Gaithersburg, MD, USA) and other reagents from Sigma (Poole, UK) unless specified. CD34<sup>+</sup> hematopoietic progenitor cells (a) and dendritic cells (b) are shown after fluorescence microscopy.

and flow cytometric sorting allows the removal of GFP-negative nontransduced cells, resulting cells show an effective transduction efficiency of 100% for the second gene. Thus the substitution of a 'test' gene for the *lacZ* can allow experiments to be performed in these cell types with a uniformly transduced population which could not reliably be achieved by other means. For example, in the case of dendritic cells, cancer immunotherapy by loading with tumor antigens followed by return to the patient has often been considered,<sup>10</sup> and in the case of hematopoietic progenitor cells we are using the system to study the control of the cell cycle in these quiescent cells by a herpes vector-mediated gene delivery approach. This kind of approach might also be applicable to other cell types for which efficient gene transfer is as yet of low efficiency.

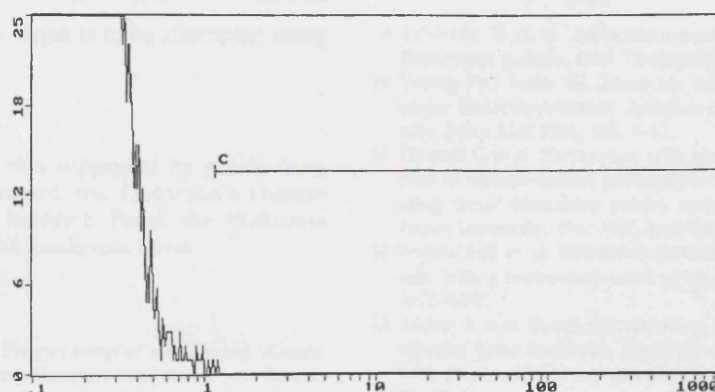
Thus, we have shown that GFP can be effectively expressed from a herpes vector both *in vitro* and in the mouse brain *in vivo*, and that 100% transduced populations of dendritic and quiescent CD34<sup>+</sup> hematopoietic progenitor cells can be produced when combined with

subsequent flow cytometric sorting. Expression of a second gene together with GFP (in this case *lacZ*) allows the second gene to be delivered at an effective efficiency of 100%, and experimental protocols requiring gene delivery and which would not otherwise be possible in these cell types could thus be performed. The use of GFP reported here combines two advantages of a herpes vector system: first, that HSV can infect these cell types at a useful level of efficiency; and second, that multiple genes can easily be expressed without exceeding the packaging capabilities of the virus. While adenovirus vectors are able to transduce dendritic cells and hematopoietic progenitor cells, multiple gene expression is hard to achieve due to the limited packaging capabilities of the virus. GFP can thus be used as an aid to increase the effective transduction efficiency of a particular cell type, as a marker for *in vivo* gene delivery, for example, where it is required to carry out electrophysiological recordings from the successfully transduced cells, or as an aid for optimisation experiments *in vitro* or *in vivo* when gene

(a)

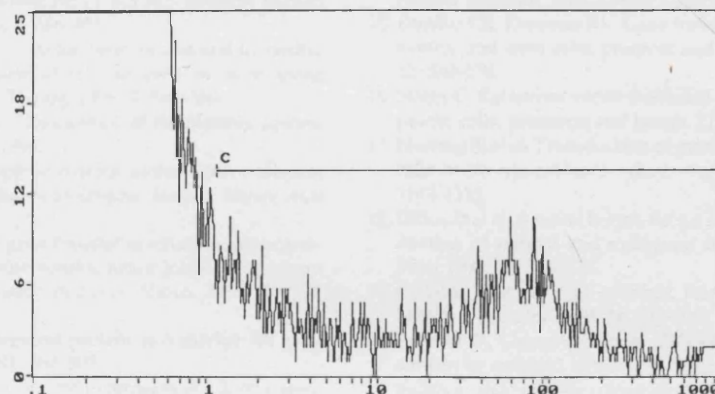
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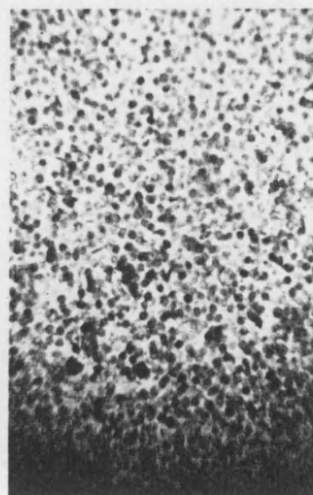
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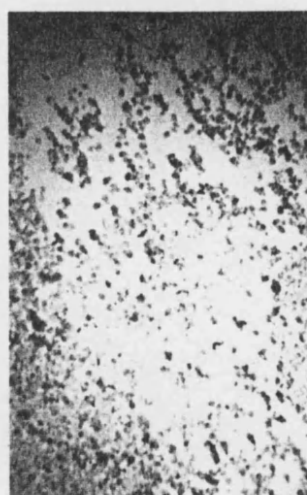


GFP

(b)



CD34+ve hematopoietic progenitor cells



Dendritic cells

**Figure 3** Flow cytometric sorting of transduced CD34<sup>+</sup> hematopoietic progenitor cells and dendritic cells. The percentage of cells expressing GFP was quantified by flow cytometry (EPICS-Elite; Coulter Electronics, Luton, UK) and GFP-positive cells then stained for lacZ activity. (a) Flow cytometric analysis for the transduction of GFP-positive, CD34<sup>+</sup> hematopoietic progenitor cells. (b) GFP-positive hematopoietic and dendritic cells after collection, fixing in 4% paraformaldehyde in PBS for 15 min and staining for lacZ activity with X-gal for 30 min in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 1 mg/ml X-gal in PBS. Photomicrographs show an effective transduction efficiency of 100% for each cell type (100% blue cells), although expression levels are variable. No blue staining can be seen with untransfected control cells after similar incubation in X-gal (not shown).

delivery to a new cellular target is to be attempted using an HSV vector.

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## Development and Optimization of Herpes Simplex Virus Vectors for Multiple Long-Term Gene Delivery to the Peripheral Nervous System

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Herpes simplex virus (HSV) has often been suggested as a suitable vector for gene delivery to the peripheral nervous system, as it naturally infects sensory nerve terminals before retrograde transport to the cell body in the spinal ganglia, where latency is established. HSV vectors might therefore be particularly appropriate for the study and treatment of chronic pain following vector administration by relatively noninvasive peripheral routes. However parameters allowing safe and efficient gene delivery to spinal ganglia following peripheral vector inoculation, or the long-term expression of delivered genes, have not been comprehensively studied. We have identified combinations of deletions from the HSV genome which allow highly efficient gene delivery to spinal dorsal root ganglia (DRGs) following either footpad or sciatic nerve injection. These vectors have ICP34.5 deleted and have inactivating mutations in *vmw65*. We also report that peripheral replication is probably necessary for the efficient establishment of latency in vivo, as fully replication-incompetent HSV vectors allow efficient gene expression in DRGs only after peripheral inoculation at a high virus dose. Very low transduction efficiencies are otherwise achieved. In parallel, promoters have been developed that allow the long-term expression of individual or pairs of genes in DRGs by using elements from the latently active region of the virus to confer a long-term activity onto a number of promoters which otherwise function only in the short term. This work further defines elements and mechanisms within the latently active region that are necessary for long-term gene expression and for the first time allows multiple inserted genes to be expressed from HSV vectors during latency.

Herpes simplex virus type 1 (HSV-1) and HSV-2 are large DNA viruses the life cycles of which are characterized by the ability to enter a lifelong latent state in sensory neurons from which reactivation of the virus can intermittently occur. During latency the HSV genome, which remains extrachromosomal, is largely quiescent, although the fact that a small part of the genome encoding the latency-associated transcripts (LATs) is transcribed during latency (38) shows that the development of gene delivery vectors allowing transgene expression during this time should be possible. During latency HSV is not naturally cleared by the immune system and infected cells remain undamaged, further showing the potential for a long-term therapeutic benefit with HSV as a vector. HSV is a large virus, potentially allowing the insertion of multiple therapeutic genes. It thus has a number of properties suggesting that it may ultimately provide an ideal vector for gene delivery to the nervous system (reviewed in references 6 and 13).

In this study we have attempted to optimize parameters allowing gene delivery to the spinal ganglia following inoculation of HSV vectors by peripheral routes, thus taking advantage of the natural life cycle of the virus, which usually infects axonal nerve terminals at peripheral sites. Neurotrophic herpesviruses such as HSV-1 and HSV-2 are unique among viruses currently under development as vectors in that they have the ability to infect axonal nerve terminals before retrograde

transport to neuronal cell bodies where latency is established. While other viruses used as vectors, such as adenovirus, adeno-associated virus, and lentiviruses, can infect neurons in vitro and in vivo, none has evolved an ability to be efficiently transported to neuronal cell bodies in this way. In the peripheral nervous system there are a number of potential applications for vectors capable of axonal transport, including the stimulation of regrowth of damaged nerves, the study and treatment of various pain states, the protection of neurons from further degeneration in, e.g., motor neuron disease, the study and treatment of various neuropathies, the study of neuronal development, and the screening of the relevance of genes implicated as being important in any of these processes by a gene delivery approach.

Considerable previous work aimed at the development of HSV vectors has already been reported (reviewed in references 6 and 13). This has been largely targeted to overcoming two inherent problems with wild-type HSV as a vector system: how to disable the virus such that it is no longer pathogenic but such that it is still able to infect and remain latent in neurons and how to maintain transgene expression during HSV latency. For the use of HSV vectors which are to be introduced into the brain by direct injection or for gene delivery to cells in culture, it has become apparent that the expression of HSV genes from the vector must be minimized to prevent toxic effects (19, 20, 21, 37, 44). Here either the regulatory immediate-early (IE) genes encoding ICP4, ICP27, ICP22, and ICP0 must be deleted or their expression must be otherwise reduced or prevented (19, 20, 21, 37, 40, 44). This prevents toxicity from the IE proteins themselves, as each is highly cytotoxic, and also from the later genes the expression of which the IE genes stimulate.

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For gene delivery to spinal ganglia in vivo, however, usually a footpad or ear pinna inoculation route has been used, which requires transport from the inoculation site to the cell body in the spine. Here various viruses have been used, including those with the nonessential genes encoding ICP6 (16), ICP0 (24), thymidine kinase (TK) (18, 32), gC (9, 15, 25), or vmw65 (11) mutated or disabled (22, 23, 28) or (by us) with ICP34.5 or ICP34.5 and vmw65 deleted (7). These viruses have each shown reasonable levels of at least short-term gene delivery and/or the ability to enter latency following inoculation of mice by the footpad or ear pinna route. All of these viruses, however, retain at least some degree of replication competence in vivo and in culture, as no essential genes have been inactivated. So far it has not been reported whether viruses incapable of replication in any cell (i.e., with an essential gene or genes mutated) allow gene delivery to spinal ganglia following inoculation by peripheral routes such as the footpad or ear pinna. A virus with ICP4 deleted (i.e., replication incompetent) has, however, given low-efficiency delivery to dorsal root ganglia (DRGs) following direct inoculation into the sciatic nerve (9), and a virus with a partial deletion in ICP0, an inactivating mutation in vmw65, and a temperature-sensitive mutation in ICP4, providing a conditionally replication-competent virus, has allowed genes to be delivered following footpad inoculation (29) (see Discussion). In that work (29) it was concluded that replication is probably not necessary for HSV to efficiently reach spinal ganglia following inoculation by peripheral routes. A primary aim of the present work, therefore, was to identify optimal combinations of mutations and further define parameters allowing efficient gene delivery to spinal ganglia by footpad inoculation or by inoculation directly into a peripheral nerve.

As discussed above, a second problem with HSV vectors has been an inability to retain expression of inserted genes during latency, as these genes under the control of most of the HSV and non-HSV promoters which have been tested, like the majority of the HSV genome during latency, become rapidly transcriptionally inactivated (see, e.g., references 22, 25, and 28). However a number of strategies allowing latent transgene expression have been reported. First, it was found that a Moloney murine leukemia virus (MMLV) promoter linked to a fragment of the LAT promoter (LAP1) (Fig. 1) and inserted in gC was able to drive expression during latency, although neither LAP1 nor the MMLV promoter alone, nor a number of other promoters linked to LAP1 or alone, allowed this to occur (25). MMLV alone inserted in ICP4 (3, 10) or in LAP1 (4), however, was active, which may be due to the proximity of these regions to the endogenous LATs in this case, in contrast to being distant from the LAT region (in gC) before (25). In other approaches it was found that LAP2 alone (Fig. 1) could give expression during latency when inserted in gC (15) (but that this expression was very weak) and that LAP2 linked to LAP1, like MMLV linked to LAP1, could also maintain latent gene expression when inserted in gC (26). Finally, it was found that insertion of an internal ribosome entry site into the 2-kb LAT allowed expression of a downstream marker gene [with a poly(A) site] during latency (23). This strategy thus retained the natural LAT promoter structure, overcoming the problem that the RNA expressed during latency (the 2-kb LAT) would not usually allow protein expression as it is usually nuclear and nonlinear in nature (36, 45).

Thus, due to the factors described above, a second aim of the work reported here was to further define parameters enabling gene expression during latency and in particular to develop systems which would allow the long-term expression of multiple genes during latency and which could be inserted at any

desired site within the HSV genome. This would take advantage of the large genome size of HSV, allowing insertion of ~15 kb of exogenous sequence and of even larger insertions if the HSV sequence is first removed as is the case in most vectors.

#### MATERIALS AND METHODS

**Cell culture and virus propagation.** Replication-competent and -incompetent viruses were propagated on BHK cells and 27/12/M:4 cells (40) (27/12/M:4 cells are BHK cells which complement mutations in ICP4, ICP27, and vmw65), respectively. Cells were cultured in Dulbecco modified Eagle medium plus 10% fetal calf serum with 800 µg of neomycin per ml and 750 µg of Zeocin per ml for 27/12/M:4 cells (40). HMBA (3 mM) was included for propagation of viruses containing the in1814 mutation (30).

**Initial promoter constructs.** Six plasmids were constructed (see Fig. 2). These contained the LAP1 promoter (HSV-1 nucleotides [nt] 118443 to 118878 [NotI-SylI] (Fig. 1)) linked to *lacZ* (from pCH110 [Pharmacia]) and inserted into a plasmid allowing homologous recombination into UL43 at the unique *NsiI* site or the same plasmid except with the MMLV long terminal repeat (LTR) (from pJ4 [NheI-KpnI] [31]) inserted between the LAP1 sequence and the *lacZ* gene and with LAP1 sequences of either HSV-1 nt 118443 to 118878 (NotI-SylI), nt 118443 to 118671 (NotI-PstI), or nt 118181 to 118768 (DdeI-DdeI). Finally, a cassette consisting of a LAP1 sequence (nt 118181 to 118768 [DdeI-DdeI]), a ~700-bp plasmid spacer sequence derived from pGem3Zf (NdeI-XbaI) (Promega), and an MMLV LTR promoter driving *lacZ* (similar to the promoter cassette reported by Lokensgard et al. [25]) was constructed and inserted into UL43 flanking regions (giving plasmid pR8c) or into a plasmid allowing recombination into the virion host shutoff protein (vhs)-encoding gene (UL41) at the unique *NruI* site (giving plasmid pR15). HSV nucleotide numbers refer to GenBank file HE1CG.

**Replication-competent viruses.** The plasmids described above allowing insertion into UL43 were inserted into virus strain 1764 (ICP34.5 and vmw65 deficient [7]) by standard techniques of homologous recombination into purified viral genomic DNA and selection of *lacZ*-expressing plaques followed by further purification and Southern blotting to confirm genome structure. The promoter-*lacZ* cassettes contained in plasmids pR8c and pR15 were also inserted into virus strains 1716, 1764, and in1814 (vmw65 deficient [1]) by homologous recombination. (The resulting virus strains generated are shown in Fig. 3.)

**Second-generation promoter constructs.** Plasmids were constructed allowing homologous recombination into the HSV-1 LAT region, using a 3.5-kb *NotI* fragment (HSV-1 nt 118443 to 122029) inserted into pGem5 (Promega). A *lacZ* gene (from pCH110, *HindIII*-*Bam*HI [Pharmacia]) was inserted between the two *SylI* sites (between nt 118878 and 119248) or the two *BbsI* sites (between nt 119674 and 121328); a cytomegalovirus (CMV) promoter (from pcDNA3 [Invitrogen])-*lacZ* cassette was inserted between the *SylI* sites and between the *BstXI* sites (nt 120220 and 120408); and a minimal CMV promoter (156-bp *SmaI*-*SmaI* fragment from pUHC13-3 [17])-*lacZ* cassette, a neuron-specific enolase (NSE) promoter (1.8 kb [14]), a minimal NSE promoter (255-base 3'-terminal fragment from the 1.8-kb NSE promoter [40]), and an MMLV promoter (from pJ4)-*lacZ* cassette were inserted between the same *BstXI* sites. These were inserted into virus strain 1764 by homologous recombination (see above). (The resulting constructs are shown in Fig. 5.)

**pR20.5 and pR20.9.** The pR20.5 cassette has previously been described (40). The pR20.9 cassette is identical except with the replacement of the CMV promoter by the MMLV LTR promoter from pJ4 (31) and of the Rous sarcoma virus promoter by the LAP1 promoter (HSV-1 nt 118181 to 118878, *DdeI*-*SylI*). These were inserted into plasmids containing the UL43 or vhs flanking regions as described above or into a plasmid containing sequences allowing recombination into US5 at the unique *SacI* site (nt 137945). (The structures of these plasmids are shown in Fig. 7.)

**Replication-incompetent viruses.** Virus strain 17+/27-/pR19lacZ has previously been described (42) and contains a CMV-*lacZ* cassette inserted into the LAT region (as shown in Fig. 5) of HSV strain 17+ from which ICP27 has been deleted. Virus strains 1764/27-/pR20.5/vhs and 1764/27-/4-/pR20.5/vhs were constructed by homologous recombination of the pR20.5 cassette inserted into vhs gene-containing flanking regions (inserted into the *NruI* site as before) into virus strains with ICP34.5, ICP27, and the LAT P2 region deleted and containing the in1814 mutation, or also with ICP4 deleted, respectively (40). Deletion of endogenous LAT P2 regions prevents recombinational instability which is otherwise caused by insertion of LAT P2 elements (contained in pR20.5) in vhs. Virus strain 1764/27-/4-/pR19lacZ was constructed as was virus strain 17+/27-/pR19lacZ, except with recombination of the CMV-*lacZ* cassette into the LAT region of the multiply deleted virus backbone described above. Here LAT P2 elements are not deleted. Replication-incompetent viruses were propagated as previously described (40).

**In vivo gene delivery.** Viruses were inoculated by the footpad route as previously described, and at various times postinoculation animals were killed and DRGs were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (for *LacZ* expression) as reported previously (7), or the X-Gal staining step was omitted for observation of green fluorescent protein (GFP) expression by fluorescence microscopy. When dual expression of GFP and *lacZ*



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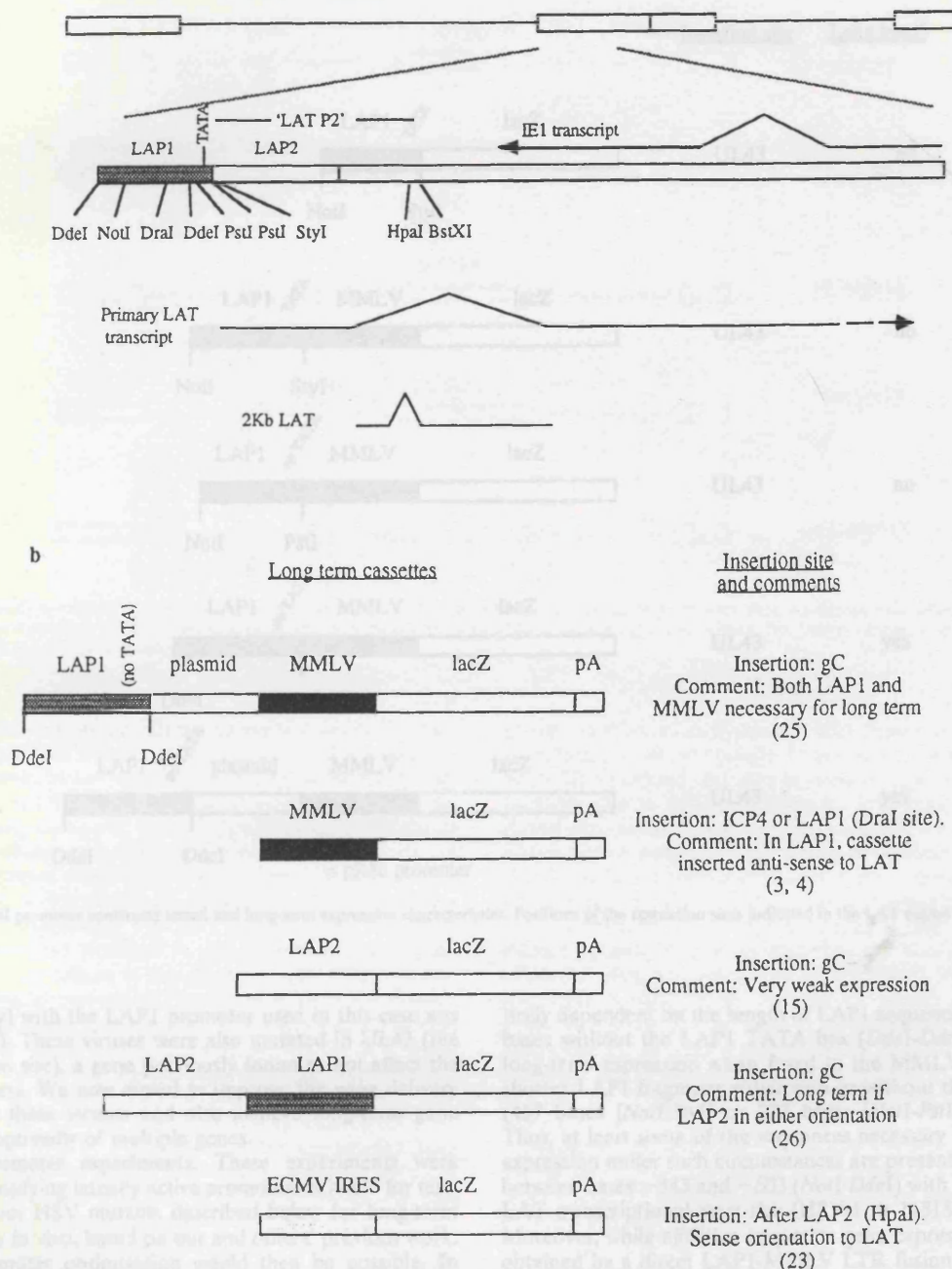


FIG. 1. The LAT region and promoter cassettes which have previously been identified as driving long-term gene expression. (a) Schematic representation of the LAT region showing relevant restriction sites, the promoter regions LAP1 and LAP2, and the LAT P2 region mentioned in the text. (b) Previous promoter cassettes giving gene expression during HSV latency. ECMV, ●●●, IRES, internal ribosome entry site.

was to be assessed, GFP fluorescence was examined prior to X-Gal staining, as this otherwise masks fluorescence from GFP. When DRGs were to be sectioned, animals were ●●● perfusion rather than post-fixed in 4% paraformaldehyde prior to further processing. For sciatic nerve inoculation, animals were anesthetized, the sciatic nerve was exposed, and 2 to 5  $\mu$ l of virus suspension was injected into the sciatic nerve using a glass micropipette. The quantity of 2 to 5  $\mu$ l was used as by this procedure not all of the vector suspension tends to remain in the nerve. Injection was thus continued (up to 5  $\mu$ l) until injection of ~2  $\mu$ l was concluded to have been achieved. Using smaller volumes did not give as reproducible results. The wounds were then closed, and animals were allowed to recover. For footpad inoculation (20- $\mu$ l inoculation volume in all cases) viruses were usually used at a titer of  $10^6$  PFU/ml, and for sciatic nerve inoculation viruses were used at  $10^7$  PFU/ml, except when otherwise indicated.

**In situ hybridization.** In situ hybridization was carried out exactly as described previously (31) except with a LAT-specific oligonucleotide probe complementary to HSV-1 nt 120691 to 120647 (5'-TAAAGGTTTACITTTGTATCTTTTCCC TGCTGTGTTGGATGTAT).

## RESULTS

We have previously found that viruses with ICP34.5 deleted or with ICP34.5 deleted and also with an inactivating mutation in vmw65 (the in1814 mutation) can allow gene delivery to DRGs following footpad inoculation but that gene expression

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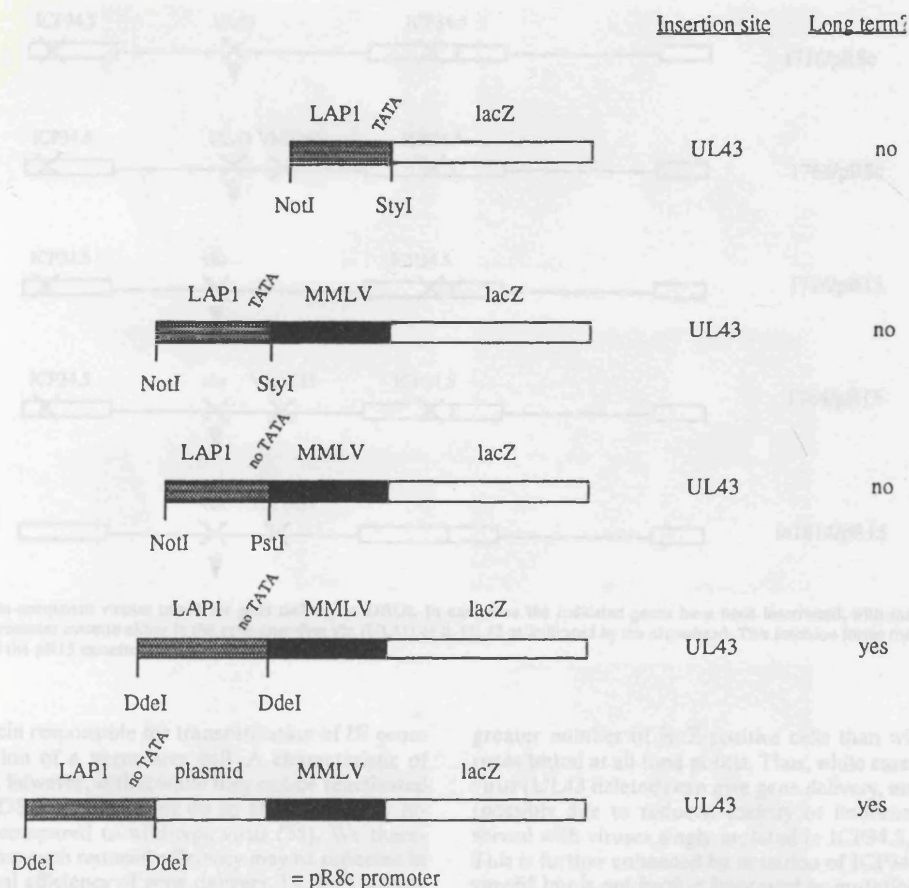


FIG. 2. Initial promoter constructs tested and long-term expression characteristics. Positions of the restriction sites indicated in the LAT region are shown in Fig. 1.

(*lacZ* activity) with the LAP1 promoter used in this case was transitory (7). These viruses were also mutated in UL43 (the *lacZ* insertion site), a gene previously found to not affect the latency process. We now aimed to improve the gene delivery efficiency of these viruses and also achieve long-term gene expression, optimally of multiple genes.

**Initial promoter experiments.** These experiments were aimed at identifying latently active promoters suitable for testing the various HSV mutants described below for long-term gene delivery in vivo, based on our and others' previous work. Further promoter optimization would then be possible. In these experiments (Fig. 2) a LAP1 promoter (437 bases, *NotI-StyI*), a LAP1 (437 bases, *NotI-StyI*)-MMLV LTR fusion, a LAP1 (223 bases, no TATA, *NotI-PstI*)-MMLV LTR fusion, a LAP1 (585 bases, no TATA, *DdeI-DdeI*)-MMLV LTR fusion, and a LAP1 (585 bases, no TATA)~700 bases of plasmid DNA-MMLV LTR fusion were tested for the long-term expression of *lacZ* when inserted into UL43. The last construct is very similar to the promoter identified as having long-term promoter activity by Lokensgard et al. (25) (Fig. 1), where ~700 bases derived from the BAG vector (34) separated the LAP1 and MMLV LTR sequences. These experiments showed (Fig. 2) that as expected, LAP1 sequences alone could not drive long-term gene expression. They also showed that, similar to the work of Lokensgard et al. (25), a LAP1-MMLV fusion could give long-term expression. However this was en-

tirely dependent on the length of LAP1 sequence used, as 585 bases without the LAP1 TATA box (*DdeI-DdeI*) could give long-term expression when fused to the MMLV LTR, but a shorter LAP1 fragment either with or without the TATA box (437 bases [*NotI-StyI*] or 223 bases [*NotI-PstI*]) could not. Thus, at least some of the sequences necessary for long-term expression under such circumstances are present within LAP1 between bases -343 and -603 (*NotI-DdeI*) with respect to the LAT transcriptional start site (HSV-1 nt 118180 to 118440). Moreover, while effective long-term gene expression could be obtained by a direct LAP1-MMLV LTR fusion, this was enhanced if the MMLV and LAP1 sequences were separated by ~700 bases of plasmid DNA. Thus, these results, as well as further defining the regions necessary for LAP1 long-term activity when combined with the MMLV LTR, also provided an appropriate promoter for the further optimization of gene delivery experiments described below.

**Deletion of *vhs* or inactivation of *vmw65* in replication-competent HSV vectors.** It was thought likely that some degree of replication competence might be necessary for the efficient establishment of latency when HSV is inoculated at peripheral sites, but for a safe and effective vector the virus should include multiple disabling mutations. Both the ICP34.5 and the *vmw65* mutation significantly reduce pathogenicity in vivo by both intracerebral and footpad inoculation but still allow some replication (1, 27). ICP34.5 is a neurovirulence factor, and *vmw65*

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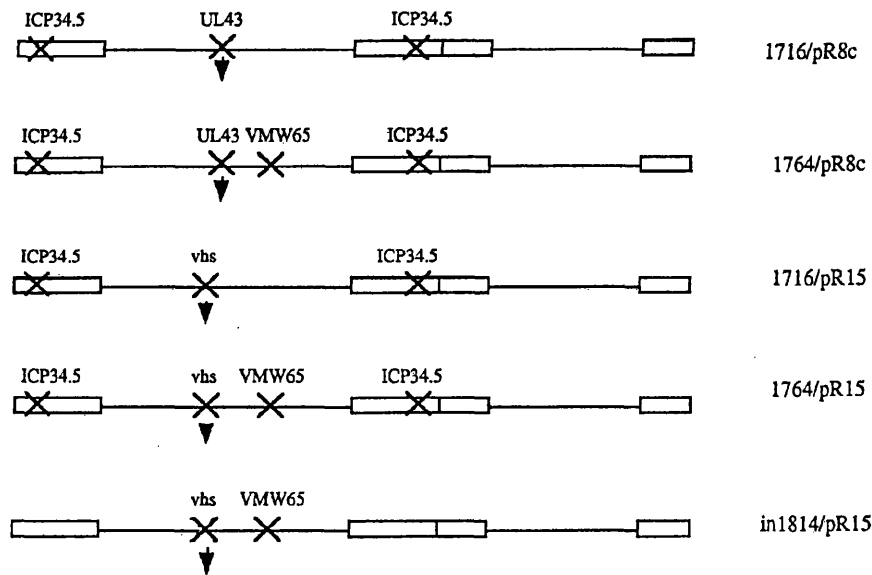


FIG. 3. Replication-competent viruses tested for gene delivery to DRGs. In each case the indicated genes have been inactivated, with insertion of a chimeric LAPI-MMLV-*lacZ* promoter cassette either in the gene encoding vhs (UL41) or in UL43 as indicated by the arrowhead. This insertion forms the pR8c cassette when inserted in UL43 and the pR15 cassette when inserted in vhs.

is the virion protein responsible for transactivation of IE genes soon after infection of a permissive cell. A characteristic of ICP34.5 mutants, however, is that while they can be reactivated from latency by DRG explant, they do so at considerably reduced efficiency compared to wild-type virus (35). We therefore speculated that such reduced efficiency may be reflected in a less-than-optimal efficiency of gene delivery, i.e., that latency establishment, which would be thought to be necessary for efficient gene expression, was also compromised. A further gene giving reduced virulence in vivo if mutated but not affecting growth in culture is that encoding vhs, (39). vhs mutants, like vmw65 mutants (12), reactivate at near-wild-type levels in DRG explant assays (39). This suggested to us that combining vhs mutations with mutations to ICP34.5 might increase the number of latent sites, and thus increase the resulting gene delivery efficiency, above those provided by mutation of ICP34.5 alone or ICP34.5 together with vmw65.

**Optimization of HSV gene deletions allowing marker gene delivery to DRGs.** Based on the rationale described above, a number of viruses with mutations of ICP34.5, vmw65, vhs, and UL43 singly and in most combinations were generated (Fig. 3). Each of these viruses contained a *lacZ* reporter gene driven by the pR8c promoter construct, which is known to give latent gene expression (Fig. 2) (called pR15 when inserted in vhs), inserted in either UL43 or the gene encoding vhs (UL41). These were then tested for *lacZ* activity in a time course of 3 days to 3 months following footpad inoculation of mice. Example DRGs (L4 and L5) from these experiments are shown in Fig. 4. Here it was shown that an enhanced efficiency of gene delivery was not accompanied by inactivation of vhs. However, mutation of ICP34.5 together with vmw65 consistently gave a

greater number of *lacZ*-positive cells than with the other viruses tested at all time points. Thus, while essentially wild-type virus (UL43 deleted) can give gene delivery, enhanced delivery (possibly due to reduced toxicity or immunogenicity) is observed with viruses singly mutated in ICP34.5, vhs, or vmw65. This is further enhanced by mutation of ICP34.5 together with vmw65 but is not further improved by mutation of vmw65 and vhs, ICP34.5 and vhs, or ICP34.5, vmw65, and vhs, where gene delivery efficiency is reduced compared to that for the single mutant viruses. It thus appears that the best gene delivery efficiency using any combination of ICP34.5, vmw65, or vhs mutations is obtained by mutation of vmw65 and ICP34.5. These viruses are also mutated in UL43 due to the *lacZ* insertion, although this does not itself appear to affect gene delivery efficiency, as insertion into US5 (another nonessential gene not affecting the latency phenotype [2]) is equally effective (see below).

**Promoter optimization.** Work by ourselves (described above) and others has shown that a number of different arrangements of LAT-derived elements, either alone or in combination with the MMLV LTR, can give transgene expression during latency (15, 25, 26). However, the mechanism of action of these elements is not yet known. It seems likely that attributes of the HSV DNA sequence, which is highly G+C rich ( $\approx 80\%$  G+C) and has an unusual dinucleotide composition (5), are involved in the shutdown of HSV and non-HSV promoters during latency and also in the stable chromatin-like structure maintained during this time (8). Thus, for the HSV LAT region to remain transcriptionally active during latency, the LAT region may take up a DNA structure different from that of the remainder of the genome, as otherwise the LAT

FIG. 4. Comparative gene delivery to DRGs using various replication-competent HSV vectors. The vectors shown in Fig. 3 were footpad inoculated into 3-week-old BALB/c mice (see Materials and Methods), and DRGs were removed and stained with X-Gal at either 3 days or 3 months postinoculation. L4 and L5 ganglia on the side of injection were removed from pairs of animals. Gene expression levels in L4 and L5 ganglia were in each case found to be similar. Not all DRGs are shown in some panels. In each case the genes inactivated in the respective viruses are indicated.



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ICP34.5



ICP34.5/vhs



3 days

ICP34.5/vmw65

3 months



ICP34.5/vhs/vmw65



3 days

vhs/vmw65

3 months

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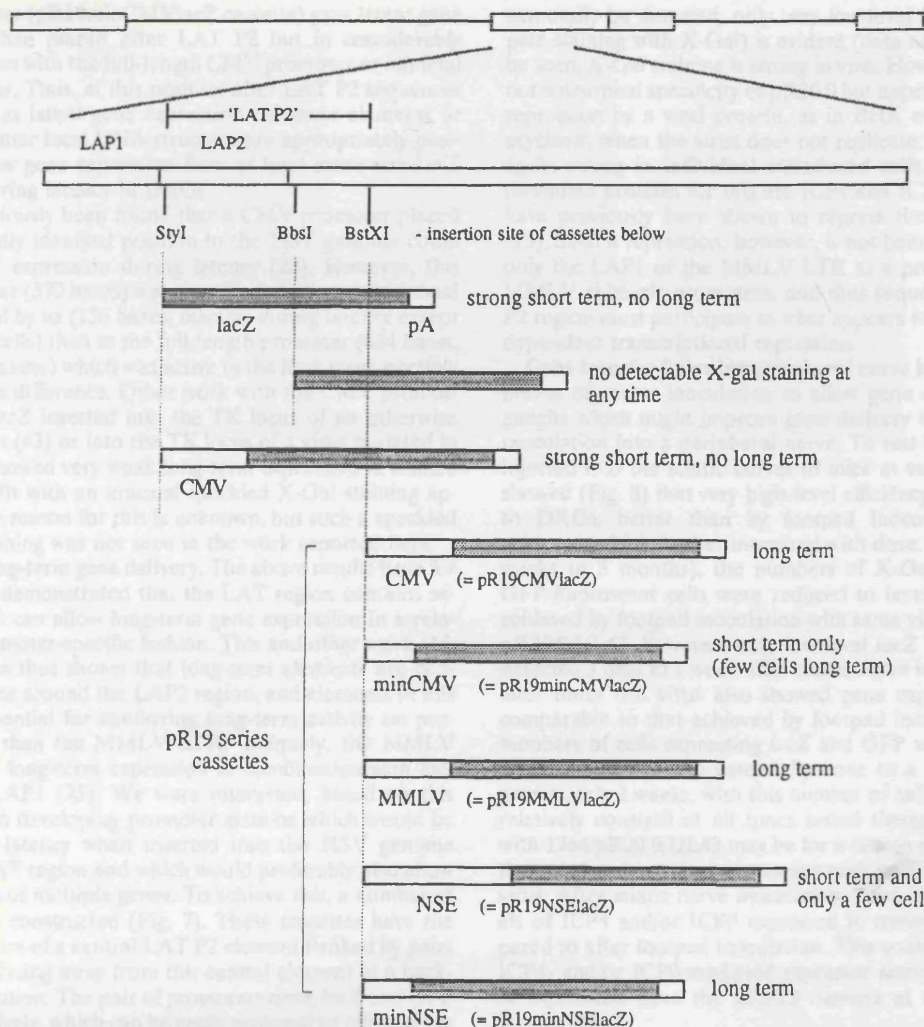


FIG. 5. Second-generation promoter constructs tested for long-term gene expression. The LAT region and relevant restriction sites are shown, indicating the positions at which the cassettes below were inserted into the HSV genome. These cassettes were inserted into the LAT regions of viruses already deficient in ICP34.5 and vmw65 (virus strain 1764) and are thus present twice per virus genome due to the diploid nature of the LAT region. LAP1 and LAP2 refer to the sequences identified by Goins et al. (15), with LAT P2 being the region used later in this study by us. The pR19 series of cassettes referred to in the text is indicated, together with a summary of the results obtained following footpad inoculation of mice with these viruses (see Materials and Methods).

region might also be expected to become transcriptionally inactive during this time. Indeed, the LAT regions of HSV-1 and other alphaherpesviruses have a considerably different dinucleotide content than the rest of their respective genomes while remaining similarly G+C rich (5). Such an altered dinucleotide content could reflect an altered DNA structure during latency. If such a hypothesis is correct, it follows that the properties of the LAT region which allow gene expression during latency would not be promoter activity related but would be structure related, and thus non-LAT promoters placed in the LAT region at an appropriate position may be able to maintain gene expression during latency due to a locally altered DNA structure caused by LAT sequences. The possibility that such a hypothesis might be correct was tested by the construction of a number of viruses (Fig. 5) (the promoters each drive *lacZ*) in which either a CMV promoter (654 bases) was placed directly after LAP1 or a CMV promoter (654 bases), a minimal CMV promoter (156 bases), an MMLV LTR

promoter, a full-length NSE promoter (1.8 kb), or a minimal NSE promoter (255 bases) was placed 1.4 kb downstream of the LAP1 TATA (after a region referred to by us as LAT P2, inserted between the two *Bst*XI sites). A *lacZ* marker gene was also placed directly after LAP1 (between the two *Syl* sites) and directly after LAP2 (15) (at the *Bst*SI site). The constructs with insertions after the *Bst*XI site form the pR19 series of cassettes referred to below.

These viruses were tested for gene expression during latency (results are also summarized in Fig. 5). This showed that neither a CMV promoter placed directly after LAP1 nor a full-length NSE promoter placed after LAT P2 could drive latent gene expression. However, a full-length CMV promoter, an MMLV promoter, and a minimal NSE promoter placed after LAT P2 (pR19CMVlacZ, pR19MMLVlacZ, and pR19minNSElacZ cassettes, respectively; the promoter insertion was made 1.4 kb downstream of the LAP1 TATA sequence) could give gene expression during latency (Fig. 6). The minimal



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CMV promoter (pR19minCMVlacZ cassette) gave latent gene expression when placed after LAT P2 but in considerably fewer cells than with the full-length CMV promoter or minimal NSE promoter. Thus, at this position after LAT P2 sequences either acting as latent gene expression enhancer elements or which might alter local DNA structure are appropriately positioned to allow gene expression from at least some non-LAT promoters during latency in DRGs.

It had previously been found that a CMV promoter placed at an essentially identical position in the HSV genome could not give *lacZ* expression during latency (22). However, this CMV promoter (370 bases) was closer in length to the minimal promoter used by us (156 bases; inactive during latency except in a very few cells) than to the full-length promoter (654 bases, including enhancer) which was active in the long term, possibly explaining this difference. Other work with the CMV promoter-enhancer-*lacZ* inserted into the TK locus of an otherwise wild-type virus (43) or into the TK locus of a virus mutated in *vmw65* (11) showed very weak long-term expression in a small number of cells with an unusual speckled X-Gal staining appearance. The reason for this is unknown, but such a speckled pattern of staining was not seen in the work reported here.

**Multiple long-term gene delivery.** The above results have for the first time demonstrated that the LAT region contains sequences which can allow long-term gene expression in a relatively non-promoter-specific fashion. This and other work (15, 23, 25, 26) has thus shown that long-term elements are contained within or around the LAP2 region, and elements in this region are essential for conferring long-term activity on promoters other than the MMLV LTR. Uniquely, the MMLV LTR can give long-term expression in combination with elements from LAP1 (25). We were interested, based on this information, in developing promoter systems which would be active during latency when inserted into the HSV genome outside the LAT region and which would preferably also allow the expression of multiple genes. To achieve this, a number of cassettes were constructed (Fig. 7). These cassettes have the general structure of a central LAT P2 element flanked by pairs of promoters facing away from this central element in a back-to-back orientation. The pair of promoters drive *lacZ* and GFP genes, respectively, which can be easily replaced by other genes when required. The pR20.5 cassette has Rous sarcoma virus and CMV promoters on either side of the LAT P2 element, and the pR20.9 cassette has an MMLV LTR and a LAP1 promoter similarly positioned. The complete cassettes are flanked by sites for the rare blunt-cutting restriction enzyme *SrfI*, allowing excision of the entire cassette and insertion into appropriate HSV DNA flanking regions and thus allowing recombination into the HSV genome at any chosen site. These cassettes were tested for gene expression during latency when inserted into either UL43 or US5 of a virus with ICP34.5 deleted and with an inactivating mutation in *vmw65* (virus strain 1764, the optimal virus identified above).

These experiments showed that latent gene expression could be obtained with pR20.5 and pR20.9 of both GFP and *lacZ* (Fig. 7 shows expression from pR20.9) following footpad inoculation of mice. Insertions into US5 (results not shown) gave results identical to those for the insertions in UL43. However, pR20.9 gave consistently more GFP- and *lacZ*-positive cells than did pR20.5, indicating the likely possibility that further elements involved in gene expression during latency are located outside LAT P2, probably in LAP1, which is included in pR20.9 but not pR20.5 (Fig. 7). Further identification of the active elements in pR20.5 and pR20.9 is under way.

pR20.9 also has the interesting property that in BHK cells when the virus is replicating, even though GFP fluorescence

can easily be detected, only very low-level *lacZ* activity (very pale staining with X-Gal) is evident (data not shown). As can be seen, X-Gal staining is strong in vivo. However, this effect is not a neuronal specificity of pR20.9 but appears to be an active repression by a viral protein, as in BHK cells together with acyclovir, when the virus does not replicate, X-Gal staining is again strong in individual transduced cells. The most likely candidate proteins for this are ICP4 and ICP0, both of which have previously been shown to repress the LAP2 promoter (15). Such a repression, however, is not been seen when using only the LAP1 or the MMLV LTR as a promoter or LAP1-MMLV chimeric promoters, and thus sequences in the LAT P2 region must participate in what appears to be a replication-dependent transcriptional repression.

**Gene transfer following peripheral nerve injection.** Another means of vector inoculation to allow gene delivery to spinal ganglia which might improve gene delivery efficiency is direct inoculation into a peripheral nerve. To test this, viruses were injected into the sciatic nerves of mice at various doses. This showed (Fig. 8) that very high-level efficiency of gene delivery to DRGs, better than by footpad inoculation, could be achieved, which further increased with dose. At later times (2 weeks to 3 months), the numbers of X-Gal-staining and/or GFP-fluorescent cells were reduced to levels similar to that achieved by footpad inoculation with same viruses. With 1764/pR20.9/UL43, however, only low-level *lacZ* activity could be detected 3 days to 1 week after sciatic nerve inoculation, but at later times this virus also showed gene expression at levels comparable to that achieved by footpad inoculation; i.e., the numbers of cells expressing *lacZ* and GFP with 1764/pR20.9/UL43 increased from essentially none to a maximum at approximately 2 weeks, with this number of cells then remaining relatively constant at all times tested thereafter. This result with 1764/pR20.9/UL43 may be for a reason similar to that for the only low-level gene expression seen in BHK cells with this virus. After sciatic nerve inoculation, there may be higher levels of ICP4 and/or ICP0 expressed in transduced cells compared to after footpad inoculation. This could, by virtue of an ICP4- and/or ICP0-mediated repressor activity, reduce levels of expression from the pR20.9 cassette at early times after inoculation.

**Gene delivery to motor neurons following peripheral inoculation.** Footpad or sciatic nerve inoculation might be expected to result in infection of motor as well as sensory axons, and thus spinal cord sections were prepared following inoculation. This showed (Fig. 9) that motor neurons in the spinal cord were also effectively transduced following peripheral inoculation with the ICP34.5- and *vmw65*-deficient viruses (Fig. 9 shows transduction after footpad inoculation with 1764/pR19CMVlacZ; see Materials and Methods), but surprisingly, the number of transduced motor neurons was not further increased by sciatic nerve inoculation. Gene delivery was limited to motor neuron cell bodies in the L4-L5 region of the spinal cord, with no evidence of transsynaptic spread to other cell types, to other regions of the spinal cord or brain stem, or to motor neurons or other cells contralateral to the injection site. This suggests that these viruses do not replicate after reaching motor neurons, although some replication would be theoretically possible with these ICP34.5- and *vmw65*-deficient viruses. Further work to optimise gene delivery to motor neurons is underway.

**Replication-incompetent HSV vectors in the peripheral nervous system.** In addition to the viruses described above, a number of viruses which cannot replicate at all in vivo were tested for gene delivery to DRGs following footpad and sciatic nerve inoculation. These viruses either had ICP27 alone de-

FIGURE 6

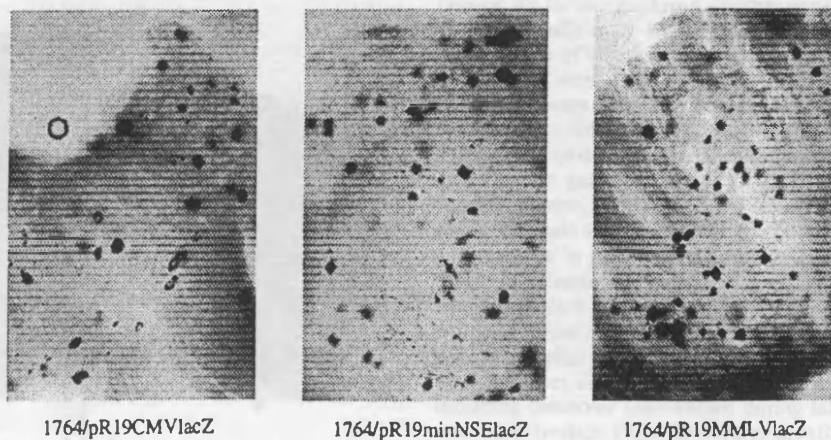


FIGURE 7

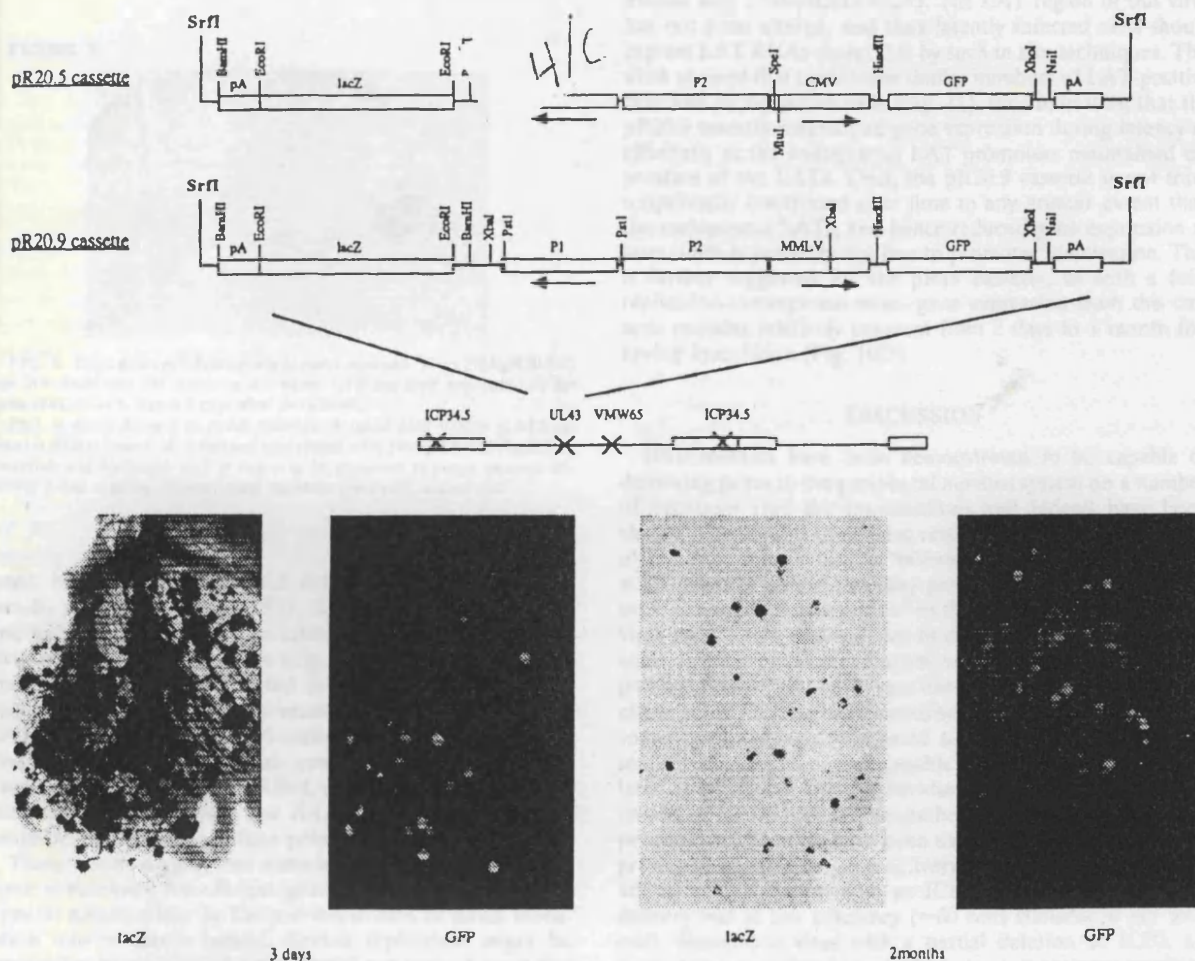


FIG. 6. Latent gene expression using second-generation promoters. X-Gal staining of DRGs was after footpad inoculation with ICP34.5- and vmw65-deficient viruses 1764/pR19CMVlacZ, 1764/pR19minNSElacZ, and 1764/pR19MMLVlacZ. Expression from each of the viruses 1 month after inoculation is shown.

FIG. 7. (Top) pR20.5 and pR20.9 expression cassettes inserted into vectors deficient in ICP34.5 and vmw65. (Bottom) Gene expression characteristics at 3 days and 2 months after footpad inoculation with the pR20.9-containing virus (1764/pR20.9/43). DRGs were either stained with X-Gal for lacZ expression or viewed by fluorescence microscopy for GFP expression. RSV, Rous sarcoma virus.



FIGURE 8

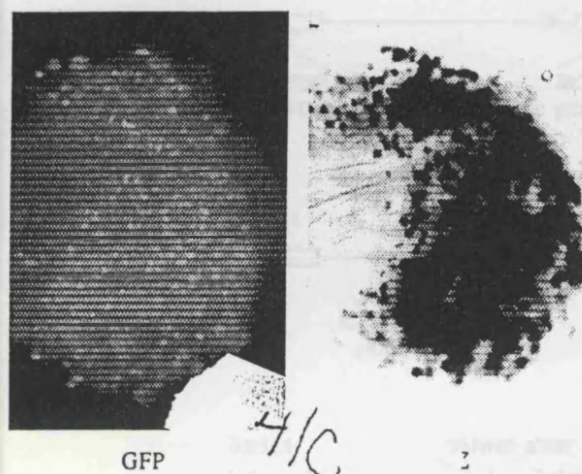


FIGURE 9

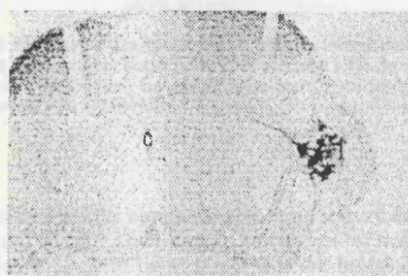


FIG. 8. Gene delivery following sciatic nerve injection. Virus 1764/pR20.5/43 was inoculated into the mouse sciatic nerve. GFP and *lacZ* expression in the same DRG (L4) is shown 3 days after inoculation.

FIG. 9. Gene delivery to motor neurons. A spinal cord section (L4-L5 region) is shown 1 week after footpad inoculation with 1764/pR19CMVlacZ (see Materials and Methods). *lacZ* is shown to be expressed in motor neurons following X-Gal staining. Sections were counterstained with neutral red.

leted, had ICP27 and ICP34.5 deleted and had the in1814 vmw65 mutation; or had ICP27, ICP4, and ICP34.5 deleted and had the in1814 vmw65 mutation (40, 42) (Fig. 10A). The virus with the pR20.5 cassette (Fig. 10A) also has the endogenous LAT P2 regions deleted to prevent recombinational instability which is otherwise caused by introduction of the LAT P2 region in the pR20.5 cassette. These results showed that following footpad or sciatic nerve inoculation at the titers usually used ( $10^8$  and  $10^7$  PFU/ml, respectively [see Materials and Methods]), only very few X-Gal- or GFP-positive cells could be identified at any time point (Fig. 10B).

These results suggest that some level of replication competence is necessary for efficient gene delivery to DRGs by peripheral routes, either by footpad inoculation or direct inoculation into a nerve. Indeed, limited replication might be expected to be necessary for the virus to penetrate through the various cell types in the footpad and also the cells surrounding the axonal bundles in the sciatic nerve. However, the finding that replication appeared to aid the virus in reaching DRGs following direct nerve injection was unexpected.

Following these somewhat disappointing results with replication-incompetent viruses as far as gene delivery was con-

cerned, we further explored whether gene delivery could occur at greater efficiency with replication-incompetent viruses if higher titers of virus were used. Here, stocks at a titer of  $5 \times 10^8$  PFU/ml were used. This gave a considerable improvement in gene delivery following sciatic nerve inoculation and some improvement following footpad inoculation (Fig. 10C). Thus, at an appropriate titer, replication-incompetent viruses can give effective gene delivery to DRGs by sciatic nerve inoculation. Moreover, gene expression continues during latency, using in this case the pR19CMV promoter cassette (Fig. 10D).

**Reduction in gene expression over time is not due to promoter inactivation.** It is evident from the data presented that while significant gene expression with the promoters used does continue in the long term, this is reduced compared to that at early times after inoculation, at least with the replication-competent viruses used. This could be for a number of reasons, including promoter inactivation during latency. To see if promoter inactivation might be occurring, rather than either clearance of the virus by immune effects or cell death caused by toxic effects of the virus, in situ hybridization was performed to detect the LAT RNAs in infected cells at 1 month after inoculation with 1764/pR20.9/UL43. The LAT region in this virus has not been altered, and thus latently infected cells should express LAT RNAs detectable by such in situ techniques. This work showed that there were similar numbers of LAT-positive cells and *lacZ*-positive cells (Fig. 11), thus indicating that the pR20.9 cassette maintained gene expression during latency as efficiently as the endogenous LAT promoters maintained expression of the LATs. Thus, the pR20.9 cassette is not transcriptionally inactivated over time to any greater extent than the endogenous LATs, and hence reduced gene expression at later times is probably not due to promoter inactivation. This is further suggested for the pR19 cassette, as with a fully replication-incompetent virus, gene expression from this cassette remains relatively constant from 2 days to 1 month following inoculation (Fig. 10D).

## DISCUSSION

HSV mutants have been demonstrated to be capable of delivering genes to the peripheral nervous system on a number of occasions (see the introduction) and indeed have been shown to be capable of altering responsiveness to painful stimuli in a gene-specific fashion following inoculation of mice with a TK-negative vector encoding preproenkephalin (43). However, a number of questions as to the necessary properties the virus must retain to allow this to occur have remained unanswered, and in most cases mutants which retain some degree of pathogenicity in vivo have been used. Thus, viruses mutated in either gC or TK have been extensively used, which, while being somewhat attenuated compared to wild-type virus, are also replication competent and capable of pathogenicity if inoculated at a sufficient titer. Otherwise, viruses with mutations in vmw65 or ICP0 which are nonpathogenic but again capable of peripheral replication have been used. Dobson et al. (9) have previously shown that gene delivery could be achieved by sciatic nerve inoculation with an ICP4 deletion virus, but this delivery was of low efficiency ( $\approx 60$  cells transduced per animal). Recently a virus with a partial deletion of ICP0, an inactivating mutation in vmw65, and a temperature-sensitive mutation to ICP4 has been shown to give relatively efficient long-term gene expression in DRGs following footpad inoculation (29). The study concluded that this strongly suggested that replication was not necessary for gene delivery following peripheral inoculation. However, this virus still retains replication competence in some circumstances, notably at temper-



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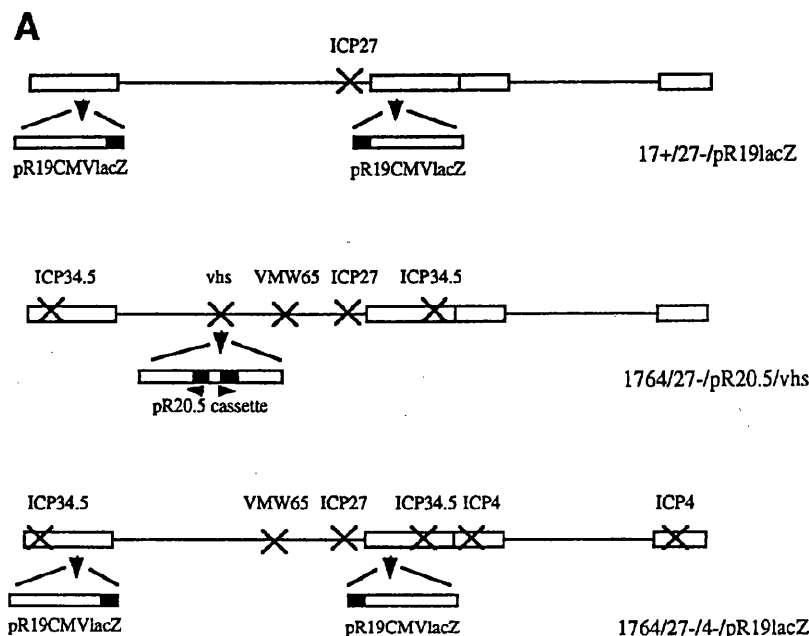


FIG. 10. Gene delivery to DRGs using replication-incompetent HSV vectors. (A) Viruses used in the experiments (see Materials and Methods; the pR19 cassette where used in these viruses contains the CMV promoter). (B) Results (*lacZ* or GFP) obtained 1 week after footpad or sciatic nerve injection with the indicated viruses. (C) Results 1 week following footpad or sciatic nerve inoculation using 1764/27-/4-/pR19CMVlacZ at a higher titer ( $5 \times 10^8$  PFU/ml rather than  $1 \times 10^7$  PFU/ml as used elsewhere for sciatic nerve inoculation). (D) Results 2 days, 1 week, and 1 month following sciatic nerve inoculation with 1764/27-/4-/pR19CMVlacZ at  $10^8$  PFU/ml.

atures below the fully nonpermissive temperature for the ICP4 mutation of  $38.5^\circ\text{C}$ . Thus, during inoculation of a mouse footpad, which is likely to be at a temperature below  $37^\circ\text{C}$ , some replication might occur, although none was detected in the reported study (29). Results with fully replication-incompetent viruses other than by sciatic nerve inoculation (9) have not been reported, and thus the question as to whether fully replication-incompetent HSV can reach spinal ganglia following peripheral inoculation has not previously been answered.

The second main area of study relevant to the development of HSV vectors for gene delivery to the peripheral nervous system has been the study and identification of promoters capable of driving gene expression during HSV latency. Thus, while expression of the LATs continues during latency, the expression of genes under the control of most other promoters does not. Recent work has shown that DNA elements from the LAT region, an internal ribosome entry site within the LAT region, or particular elements from the LAT region together with the MMLV promoter (but not other promoters) can allow a heterologous gene to be expressed in the long term (see the introduction), but the precise requirements for long-term gene expression or the mechanism for promoter shutdown which otherwise occurs has remained unknown.

The work presented here aimed to identify safe, nonpathogenic vectors which could allow gene delivery to spinal ganglia following peripheral inoculation and such that gene expression continued during HSV latency. Thus, the work on promoters aimed to identify the general principles required for long-term gene expression, particularly such that expression cassettes could be used outside the LAT region and such that multiple genes could be expressed in the long term.

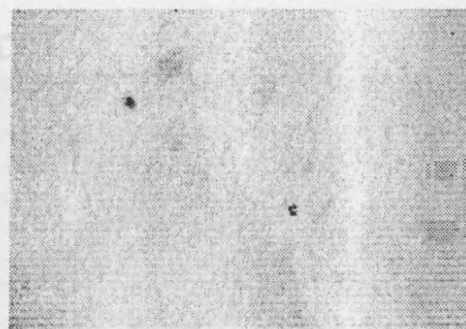
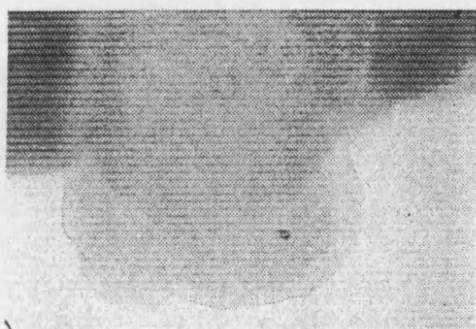
For these reasons we therefore tested either a number of HSV mutants which were attenuated by the introduction of

mutations which prevent pathogenicity in vivo, but which do not completely prevent replication (combinations of mutations in ICP34.5, vmw65, and vhs), or various viruses with mutations in ICP27 and/or ICP4 which cannot replicate in any cell (unless cells are artificially engineered to express ICP27 and/or ICP4). The first set of mutants were used because it was known that each could establish latency effectively, but each also reduced pathogenicity in vivo considerably. The highest gene delivery efficiency was achieved by the use of viruses with the combined deletion of ICP34.5 and an inactivating mutation in vmw65, by either footpad or sciatic nerve inoculation. A number of fully replication-incompetent viruses were essentially incapable of gene delivery to DRGs by either of these routes, surprisingly even when directly injected into the sciatic nerve, unless high virus titers were used. With high virus titers efficient long-term gene delivery could be achieved with replication-incompetent viruses, but only when mice were inoculated in the sciatic nerve and not by footpad inoculation. Thus, it appears that some degree of replication competence is necessary for HSV to usually reach spinal ganglia and enter latency, as in natural infections a high virus titer such as used for these experiments ( $1 \times 10^8$  to  $5 \times 10^8$  PFU/ml) would not be expected. These results conflict with the conclusions of Marshall et al. (29), who concluded that replication is probably not necessary for HSV to efficiently reach spinal ganglia following peripheral (footpad) inoculation. However, as the virus used by Marshall et al. can replicate at below  $37^\circ\text{C}$ , some limited replication may have occurred following footpad inoculation (although none was detected), possibly explaining this difference.

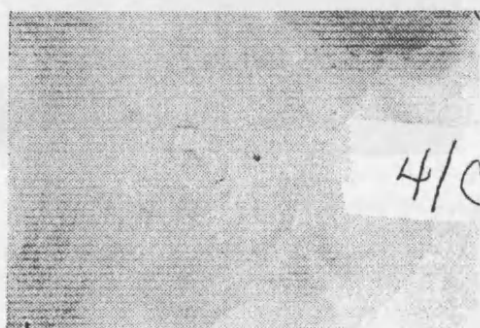
The present work has therefore identified optimized, safe, and nonpathogenic backbone viruses for gene delivery to the peripheral nervous system by peripheral injection routes. These viruses are safe, because they cannot under any circum-

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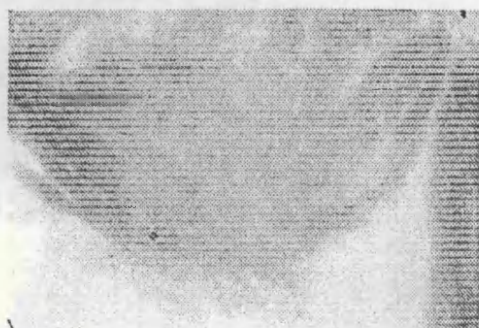
**B**



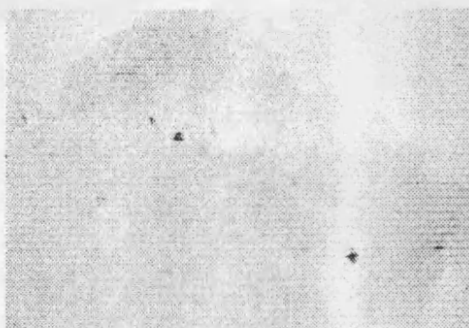
17+/27-/pR19lacZ



1764/27-/pR20.5/vhs



Footpad



Sciatic nerve

1764/27-/pR19lacZ

FIG. 10—Continued.

stances revert to a wild-type phenotype, as mutations in genes such as ICP34.5 and vnm65 cannot be repaired by recombination during growth in culture (the mutated genes are not included in the cells for growth), and they are nonpathogenic because multiple genes reducing or preventing pathogenicity have been mutated. Fully replication-incompetent viruses can also be used for gene delivery to spinal ganglia by direct intranerve injection at a sufficient titer, and these may be con-

sidered to have an improved safety profile if gene therapy (for example, for chronic pain) is to be considered for human use.

Our studies aimed at identifying parameters allowing promoters to remain active during latency have shown that the LAT region has the capability to allow long-term gene expression in a relatively non-promoter-specific fashion. Thus, the LAT region as a whole can confer a long-term activity to the LAPI promoter itself, a minimal NSE promoter, a CMV pro-



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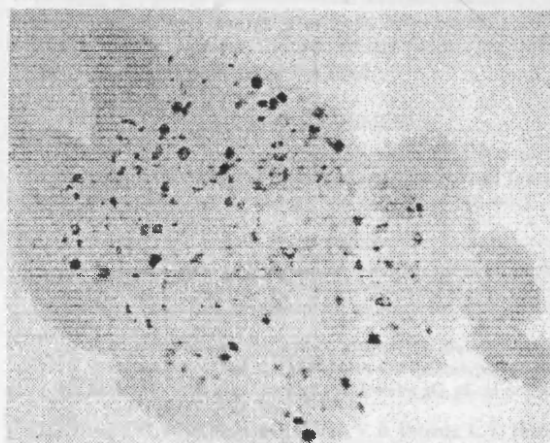
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C



Footpad

D

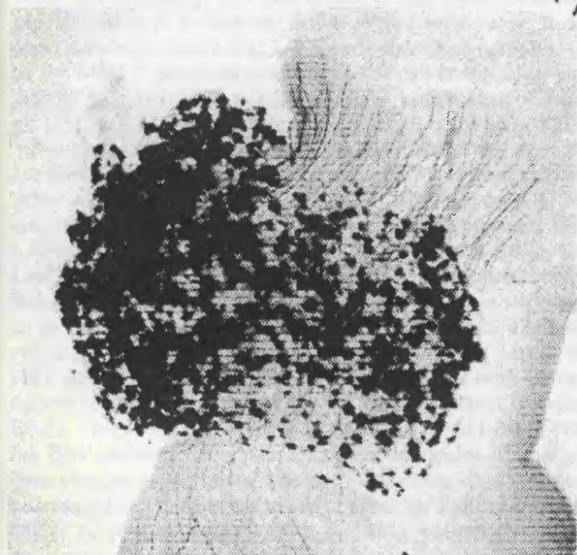


2 days

4/C



1 week



Sciatic nerve



1 month

FIG. 10—Continued.



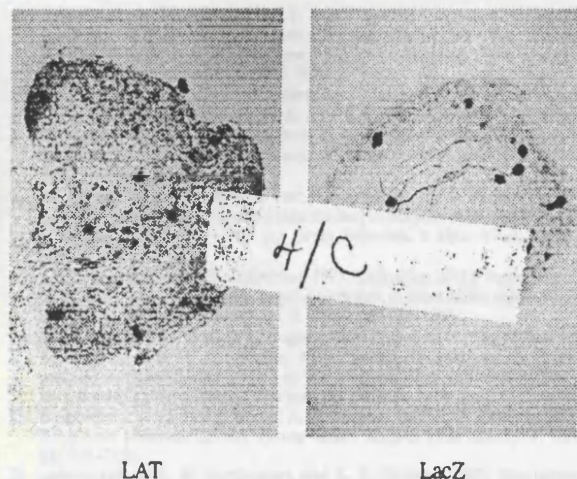


FIG. 11. LAT and *lacZ* are expressed in similar numbers of cells with 1764/pR20.9/43. Mice were footpad inoculated, and 1 month later DRG sections were either probed for LAT expression (see Materials and Methods; section was counterstained with thionin) or stained with X-Gal (section was counterstained with neutral red).

moter, and an MMLV promoter. This suggests that the LAT region might act to locally remodel the chromatin, allowing an altered DNA structure compared to the rest of the HSV genome during latency, and thus allows at least some promoters placed within it to remain active in the long term. It has previously been shown that LAP1 can confer long-term activity to the MMLV promoter and that LAP2 can confer long-term activity to LAP1, both outside the LAT region (inserted into gC [25, 26]). We have shown that sequences between positions -343 and -603 with respect to the LAT transcript start site are necessary for this LAP1-mediated activity, further defining active elements necessary for long-term gene expression. We are currently identifying active regions in and around both LAP1 and LAP2/LAT P2. Previous work had also shown that LAP2 could confer a long-term activity on LAP1 in a direction-independent fashion (26). Combining these studies suggested to us that promoter cassettes might be designed to allow the expression of pairs of genes from non-LAT sites within the HSV genome by placing pairs of promoters in a back-to-back orientation away from a centrally positioned element including LAP2. This was found to be the case, for the first time providing HSV vectors allowing the long-term expression of multiple heterologous genes during latency. Thus, while HSV has often been suggested to have advantages over other vectors due to its ability to package large inserts and thus potentially deliver multiple genes, this capability has not been able to be exploited in the long term, as only individual genes have previously been able to be expressed during latency.

Thus, overall the present work has further defined parameters allowing gene delivery to spinal ganglia by peripheral routes and has provided nonpathogenic, safe viruses suitable for this purpose. It has also further defined parameters allowing transgene expression during latency and provided promoter cassettes allowing the long-term expression of multiple heterologous genes. These cassettes can be inserted at non-LAT sites within the HSV genome. Such vectors have a number of potential uses in the study of processes occurring in or mediated via spinal ganglia. These include the study of pain states, which might ultimately allow the development of a gene therapy approach to long term analgesia, or the identification

and characterization of genes important in these processes. The ability to deliver multiple genes can allow either the delivery of a marker gene together with the gene of interest or the delivery of pairs of genes such that interactions can be studied. Such approaches have not previously been possible using HSV or other vectors unless multiple viruses (which may not necessarily infect the same cell) are used.

#### ACKNOWLEDGMENTS

J. Palmer and R. Branton contributed equally to this work. We are grateful to the Medical Research Council and International Spinal Research Trust for the support of parts of this work.

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