DEVELOPMENT OF HSV-1 VECTORS FOR APPLICATIONS IN THE PERIPHERAL NERVOUS SYSTEM

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For Mum, Dad and Rob,
with all my love
ABSTRACT

Herpes Simplex Virus (HSV) is potentially an ideal vector for gene therapy in the peripheral nervous system for a number of reasons. HSV is neurotrophic with a life cycle that involves retrograde transport of the virion from peripheral nerve terminals to sensory neuron cell bodies. Thus, for gene delivery to cell bodies in dorsal root ganglia there would be no need for vector administration by an invasive surgical route. In addition HSV has evolved the ability to enter life long latency in the nuclei of infected sensory neurons, providing the potential for very long-term transgene expression. This study aimed to identify and optimise the use of attenuated HSV-1 vectors that are non-pathogenic, non-cytotoxic and able to express transgenes during both lytic and latent infection. Several vectors deleted for various combinations of non-essential and/or essential virus genes were designed and evaluated and optimal replication, competent and incompetent vectors for peripheral gene delivery were identified. In addition, transgene expression cassettes consisting of different promoter/reporter gene arrangements were tested for their ability to express single or multiple genes in the long-term. Using this information, optimised vectors containing either the neuropeptide galanin or Cre recombinase were constructed for use in nerve regeneration studies or conditional gene expression respectively. In further studies aimed at developing regulatable gene expression systems for HSV, vectors were generated containing a number of ligand inducible transgene expression cassettes. Of these, the ecdysone regulatable system was found to be most promising and replication competent and incompetent ecdysone inducible vectors were constructed and found to give regulated gene expression in various circumstances. Overall the work described in this thesis resulted in the successful development of HSV vectors for the PNS and the development of regulatable HSV gene expression systems which are highly promising for future work.
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Many thanks to Rob Coffin and David Latchman for giving me the opportunity to work on this project and for the advice over the years.

Huge, huge thanks go to Dr P., who not only contributed towards and helped me achieve much of the work seen in this thesis, but who also taught me much of what little neuroscience I know today. James thanks also for the never ending supply of advice, gossip and for always being around for a long coffee. Special thanks go to Jill and Caroline who gave me advice from the beginning, both in terms of work and pleasure. Thanks for your friendship and for always being around. Many thanks also go to my fish-man Kevin, the "sweetest pea" of us all. Thank-you for your support, advice, lots of laughter, and a brilliant car service, even if I do abuse it from time to time. Big thanks to Suzanne for the copious technical advice and for having to put up with me, and my mess, on the bench next to hers! Thanks also go to the many members of the big and little labs, plus Biovex, who have made the lab a fun place to work and the 'King and Queen' an attractive after work venue.

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Statement of Conjoint Work

- Work in chapters 3 and 4 of this thesis was performed jointly with Dr Janes Palmer, Biovex, London.

- Galanin Immunohistochemistry (chapter 7) was performed by Dr Fiona Holmes, University of Bristol, Bristol.

- PCR to genotype ROSA-26 mice (chapter 7) was performed by those in the Department of Anatomy, UCL, London.
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
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<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>β-Gal</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>β-Geo</td>
<td>β-Galactosidase/neomycin fusion</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BmEcR</td>
<td><em>Bombyx mori</em> ecdysone receptor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DB-EcR</td>
<td>Drosophila/Bombyx ecdysone receptor</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco' modified Eagle's media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>E</td>
<td>Early gene</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EcR</td>
<td><em>Drosophila melanogaster</em> ecdysone receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EFalpha-1EP</td>
<td>Elongation factor alpha-1 enhancer/promoter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>E/GRE</td>
<td>Ecdysone/gluccocorticoid response element</td>
</tr>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>EHV</td>
<td>Equine herpes virus</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<td>FGM</td>
<td>Full growth media</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>Glycprotein</td>
<td>Glycoprotein</td>
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<tr>
<td>GAL4</td>
<td>Yeast DNA binding domain</td>
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<tr>
<td>GAP-43</td>
<td>Growth associated protein-43</td>
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<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GL-VP</td>
<td>Progesterone responsive transactivator</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced saline salt</td>
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<tr>
<td>HEBES</td>
<td>HEBES transfection buffer</td>
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<tr>
<td>HEL</td>
<td>Human embryonic lung cells</td>
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<td>HeLa</td>
<td>Human cervical adenocarcinoma cell line</td>
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<td>HCF</td>
<td>Host cell factor</td>
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<tr>
<td>hCMV</td>
<td>Human cytomegalovirus</td>
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<tr>
<td>hEGF</td>
<td>Human epidermal growth factor</td>
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<tr>
<td>hGH</td>
<td>Human growth hormone</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HMBA</td>
<td>Hexamethylene bis-acetemide</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>HSDNA</td>
<td>Herring sperm DNA</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>HSV-TK</td>
<td>Herpes simplex virus thymidine kinase</td>
</tr>
<tr>
<td>IAA</td>
<td>Isoamyl alcohol</td>
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<tr>
<td>ICP</td>
<td>Infected cell protein</td>
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<td>IE</td>
<td>Immediate early gene</td>
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<tr>
<td>IRL</td>
<td>Internal repeat long</td>
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<td>IRS</td>
<td>Internal repeat short</td>
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IRES Internal ribosome entry site
ISH In situ hybridisation
ISRT PCR In situ reverse transcription polymerase chain reaction
ITR Inverted terminal repeat
Kb Kilobase
kDa Kilo Dalton
L Late gene
LacZ β-galactosidase
LAP1 Latency associated promoter 1
LAP2 Latency associated promoter 2
LATP2 Latency associated promoter P2
LAT Latency associated transcript
LTE Long term element
LTR Long terminal repeat
MAR Matrix attachment region
‘MMTV Murine mammary tumour virus
MMLVLTR Moloney murine leukaemia virus long terminal repeat
MOI Multiplicity of infection
MurA MuristeroneA
ND7 Mouse neuroblastoma DRG fusion cell line
NGF Nerve growth factor
nls Nuclear localisation signal
nm Nanometre
NP40 Nonidet P40
nt Nucleotide
NT-3 Neurotrophin-3
ORF Open reading frame
p Plasmid
PΔHSP Heat shock protein minimal promoter
pA Polyadenylation signal
PAGE Polyacrylamide gel electrophoresis
PBL Peripheral blood lymphocyte
PBS Phosphate buffered saline
PBST Phosphate buffered saline tris
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>pi</td>
<td>Postinoculation</td>
</tr>
<tr>
<td>PonA</td>
<td>PonasteroneA</td>
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<tr>
<td>PR-LBD</td>
<td>Progesterone-receptor ligand binding domain</td>
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<tr>
<td>RCR</td>
<td>Replication competent retrovirus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RSV</td>
<td>Rous sarcoma virus promoter</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTPCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>rtTA</td>
<td>Reverse tetracycline transcriptional transactivator</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid-X-receptor</td>
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<tr>
<td>RU 486</td>
<td>Mifepristone</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free media</td>
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<tr>
<td>SIN</td>
<td>Self-inactivating virus</td>
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<td>SIV</td>
<td>Simian immunodeficiency virus</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′N′-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TetR</td>
<td>Tetracycline repressor</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline response element</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxyl)aminomethane</td>
</tr>
<tr>
<td>TRL</td>
<td>Terminal repeat long</td>
</tr>
<tr>
<td>TRS</td>
<td>Terminal repeat short</td>
</tr>
<tr>
<td>tTA</td>
<td>Tetracycline controlled transcriptional transactivator</td>
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<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>UL</td>
<td>Unique long</td>
</tr>
<tr>
<td>US</td>
<td>Unique short</td>
</tr>
<tr>
<td>USP</td>
<td>Ultraspiracal gene</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey cell line</td>
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<tr>
<td>VgEcR</td>
<td><em>Drosophila melanogaster</em> ecdysone receptor</td>
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<tr>
<td>VgRXR</td>
<td>Ecdysone controlled transcriptional transactivator</td>
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<tr>
<td>vhs</td>
<td>Virion host shut off protein</td>
</tr>
<tr>
<td>VP</td>
<td>Herpes simplex virus virion protein</td>
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<tr>
<td>VSVG</td>
<td>Vesticular stomatitis virus G protein</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>X-Gal</td>
<td>4-chloro, 5-bromo, 3-indolyl-β-galactosidase</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
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CHAPTER 1:

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1.1. GENE THERAPY – AN OVERVIEW.

In the last few decades our increased awareness of the inherited nature of many diseases has meant that we are now able to understand the mechanisms involved in their pathologies. The recent announcement of a ‘working draft’ of the human genome sequence (Macilwain 2000), provides us with a wealth of information and perhaps the most exciting achievement in scientific terms since the work of James Watson and Francis Crick in identifying the structure of DNA (Watson and Crick 1953). In terms of gene therapy the completion of the human genome sequence will increase the potential of the field significantly. Geneticists predict that the number of genes likely to be identified, based on the number of protein coding regions, could be somewhere between 35,000 and 150,000 (Butler and Smaglik 2000). Once characterised these are likely to provide a wealth of potential new targets for traditional drug or gene therapy.

Gene therapy can be defined as the introduction of genetic material into cells to bring about a therapeutic effect. So far gene therapy has been limited to somatic gene therapy whereby the genetic material is inserted into diploid cells of an individual. In this case none of the acquired genetic material is passed between generations. Germline gene therapy is so far ethically unacceptable, but might be regarded as the ultimate form of gene therapy. So far somatic gene therapy can be divided into three general areas based on the route of gene administration; ex vivo, and direct or indirect in vivo (Drew and Martin, 1999). The ex vivo approach involves explant of a patient's cells, for example stem cells or lymphocytes, followed by culture in vitro. The cells are then manipulated and transduced with the therapeutic gene and finally re-implantated back into the patient. This appears to be a relatively simple procedure and in theory should minimise problems with immunogenicity and rejection, which might otherwise be associated with the use of allogeneic cells. However, a frequent occurrence and significant limitation is the low level of cell viability that is found after re-implantation. Direct in vivo gene delivery involves direct gene transfer to the site of interest and is currently an area in which
considerable research activity is underway. Most of the commonly used vectors (see later) have been used in this approach to transfer genetic material directly into target diseased tissue, although the use of naked DNA itself has also been explored. *In situ* gene delivery removes any need for targeting of vectors but at present the efficiency of transduction is variable and sometimes problematic. Indirect *in vivo* delivery refers to the administration of genetic material by peripheral routes, for example into the blood stream, followed by targeting of the vector to the tissue requiring therapy. This approach is theoretically the most straightforward, based on the simplicity of administration, but successful targeting of therapeutic doses to the appropriate tissue is a major problem.

1.2. GENE THERAPY FOR MONOGENIC DISEASES.

Many disease states arise from chromosomal abnormalities that are either inherited or acquired. These diseases can be monogenic or polygenic. Conventional disease treatment often treats only the symptoms of the disease. However to completely cure a disease, it is necessary to correct any abnormalities which might be genetically based i.e. gene therapy would be necessary. Ideal targets for gene therapy are thus monogenic disorders, as these diseases are more easily understood and only a single gene would need to be introduced/replaced (see figure 1.1.). Currently gene therapy is aimed at gene augmentation, where-by a normal copy of a gene is introduced into the cell such that it can produce sufficient quantities of the correct gene product and can compensate for the lack of expression of the mutant host gene. Using strategies of this kind when the mutant gene has a dominant negative effect would be technically difficult. Thus disease targets have been limited to mutant genes which do not have an active negative effect such as cystic fibrosis or the severe combined immunodeficiency diseases, (X-linked SCID [SCID-X1] and adenosine deaminase deficiency-SCID [ADA-SCID]) (Cheng *et al.* 1990; Hirschhorn 1990; Noguchi *et al.* 1993). In each of these cases the disease-causing gene has been identified (see table 1.1.) allowing the gene augmentation approach to be used.
<table>
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<th>Disease Causing Gene</th>
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<tbody>
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<td>Cystic fibrosis</td>
<td>Cystic fibrosis transmembrane conductance regulator (CFTR)</td>
</tr>
<tr>
<td>Haemophilia A</td>
<td>Blood Clotting Factor VIII</td>
</tr>
<tr>
<td>Haemophilia B</td>
<td>Blood Clotting Factor IX</td>
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<tr>
<td>X-linked SCID</td>
<td>Gamma chain of IL2, IL4, IL7, IL11 and IL15 receptors</td>
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<tr>
<td>ADA SCID</td>
<td>Adenosine Deaminase</td>
</tr>
<tr>
<td>Gauchers disease</td>
<td>Glucocerebrosidase</td>
</tr>
<tr>
<td>Duchenne muscular dystrophy</td>
<td>Dystrophin</td>
</tr>
<tr>
<td>Familial Hypercholesterolemia</td>
<td>Low density lipoprotein (LDL) receptor</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>Phenylalanine hydroxylase</td>
</tr>
<tr>
<td>Huntingdons chorea</td>
<td>Huntingtin</td>
</tr>
</tbody>
</table>


Gene Therapy Protocols by Disease

Figure 1.1. Gene therapy protocols by disease. Adapted from http://www.wiley.co.uk/genetherapy/vectors.html
1.2.1. Treatment of Monogenic Disorders with Viral Vectors.

Cystic Fibrosis is caused by a dysfunction of an epithelial cell surface chloride channel (CFTR: cystic fibrosis transmembrane conductance regulator) (Cheng et al. 1990). Sufferers have a life expectancy of approximately 30 years, death resulting from effects in the respiratory epithelium. The respiratory epithelium provides an apparently easy and available target for gene therapy. Indeed viral and other vectors have been extensively tested in such approaches. Adenoviral vectors seem the instinctive choice for gene therapy in the lung as they have a natural tropism for airway epithelia (Shenk 1996). The first reported clinical trial using a recombinant adenovector was reported in 1993 (Zabner et al. 1993). Three CF patients showed a correction in the defective chloride channel, in a small area of the nasal epithelium, after administration of the Ad(CFTR) vector. Since then there have been a number of further published clinical trials for CF using first generation E1 deleted adenoviruses (Bellon et al. 1997; Boucher et al. 1994; Crystal et al. 1994). These phase one studies have looked at immunological and inflammatory response to the E1 deleted vectors, although evidence for efficiency has been limited.

ADA-SCID was the first disease to begin treatment in a gene therapy phase 1 clinical trial (Blaese et al. 1995). The disease is caused by the defective expression of the enzyme ADA, which plays a role in the purine salvage pathway (Hirschhorn 1990). The disease is characterised by a block in T-lymphocyte differentiation in the thymus. Here an ex-vivo approach in which autologous T-lymphocytes were transduced with a retroviral vector containing the human ADA gene was used. After T-lymphocyte re-infusion, which was repeated on a monthly basis over the course of two years, the two patients in the trial became normalised in their peripheral blood T-lymphocyte (PBL) population. This was the first success for a gene therapy protocol. A similar trial was also performed in Japan (Onodera et al. 1998). Autologous T-lymphocyte infusions were given and a year after the last infusion 10-20% of the patients PBL population contained the transduced ADA gene. More recently a group in Paris (Cavazzana-Calvo et al. 2000) successfully treated 3 babies suffering from X-linked SCID.
Haemophilia A and B are both X-linked inherited bleeding conditions, caused by defects in the blood clotting factors VIII and IX respectively (Martin and Drew, 1999). Untreated the conditions are lethal. Successful treatment only requires that the missing blood clotting factors are present in the plasma at about 4-5% of normal circulating levels (Scriver 1989). As the protein is secreted it may be supplied from almost any tissue, such as muscle, blood or fibroblast cells. The first reported gene therapy clinical trial for haemophilia B was in 1993 (Lu et al. 1993). Here a group in China adopted an ex vivo approach whereby a retroviral vector encoding factor IX was used to transduce autologous fibroblasts from two patients. After 3-4 administrations over a period of four months, one of the two patients showed an increase in factor IX antigen and blood clotting activity. The results of this trial provided the first real indications that human gene therapy could be effective in practice.

1.3. GENE THERAPY FOR ACQUIRED DISORDERS.

Although monogenic inherited genetic disorders were the first targets for gene therapy, today the field is dominated by clinical trials in cancer patients (See http://www.wiley.co.uk/genetherapy/vectors.html) (see figure 1.1). Cancer might seem an unusual choice for gene therapy strategies, the classical idea of which is that of the insertion and expression of a normal copy of the mutated gene. Cancer generally arises as the culmination of a multi-step process involving many genes. Currently there are many cancer gene therapy approaches in development (reviewed in Roth and Cristiano 1997; Vile et al. 2000)). These include the use of recombinant vaccines for immunotherapy, the protection of bone marrow from cytotoxicity during chemotherapy (via transduction of drug resistance genes to bone marrow stem cells) and the delivery of enzymes which convert inactive prodrugs into active drugs. The genes delivered include those for cytokines and tumor suppressor genes.

Herpes simplex virus-1 (HSV-1) thymidine kinase (HSV-TK) has the ability to phosphorylate a range of nucleosides and their analogues whereas human TK cannot (French-Anderson 1998). Because of this characteristic it has been much used in suicide gene therapy for cancer patients. Currently there are phase I/II clinical trials underway using for example, adenoviral vectors (Morris
et al. 2000; Trask et al. 2000). Gene therapy employing TK usually involves the administration of a vector containing the TK gene to the tumor cells directly or into the blood vessels supplying the tumor. In theory after systemic administration of a pro-drug such as acyclovir or gancyclovir, only cells to which the HSV-TK gene had been delivered will phosphorylate the drug to give cytotoxic acyclovir or gancyclovir triphosphates respectively (French-Anderson 1998). These are incorporated into newly synthesised DNA resulting in cell death. This strategy relies on the selective uptake and compartmentalisation of the vector by malignant tissue. Similar strategies involving transfer of the cytosine deaminase gene, which gives sensitivity to 5-fluorocytosine, to tumor cells have also been tested using adenoviral vectors (Shirakawa et al. 1999; Ichikawa et al. 2000).

1.4. GENE THERAPY VECTORS.

To facilitate the entry of therapeutic genes into cells a delivery vehicle is required. This vehicle is termed a vector and vectors for gene therapy can be broadly divided into non-viral and viral systems. The optimal vector should possess a number of characteristics and these are defined in table 1.2. However, to date no single vector system possesses all of the desirable attributes. The choice of currently available vector will thus depend upon the disease being treated and the particular requirements for gene delivery.

1.4.1. Non Viral Vectors.

At present non-viral vectors have been less successful than viral vectors in terms of efficiency, particularly in vivo. However, the potential safety issues surrounding the use of virus-derived vectors, coupled with the ease of production of non-viral systems, makes them an attractive alternative. Non-viral systems that are currently being used include the transfer of naked plasmid DNA and use of cationic liposome or polymer complexes (Li and Huang 2000). Tissue directed gene expression using naked DNA can be achieved by electroporation (Rols et al. 1998), microinjection or biolistic techniques (Yang et al. 1990). However a delivery vector, such as a liposome or polymer complex, would usually be required for indirect administration routes such as intravenous delivery.
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The vast majority of non-viral vectors are based on cationic liposome complexes (lipoplexes) (Miller AD 1999). The lipoplex consists of a positively charged lipid complex, bound to nucleic acid. The complex enters the cell by endocytosis brought about by the interaction between the positively charged lipid and the negatively charged cell surface proteoglycans. Thus, cells deficient in proteoglycans are difficult to transfect. At this point the lipoplex in the endosome can either become trapped in a late endosome or the nucleic acid can be released into the cytoplasm. However both liposome and polysome based complexes (which are largely similar in their method of gene delivery) have some enormous problems (Li and Huang 2000; Miller 1999). The complexes tend to aggregate before endocytosis and/or are unstable in the presence of serum proteins. The uptake procedure can also take hours and once inside the cell most of the complexes probably remain within late endosome particles and are thus unable to direct gene expression. A number of methods have been employed to circumvent these problems such as the ‘complexing of the liposomes with polyethylene glycol (PEG), in order to stop aggregation (Hong et al. 1997). Another potential disadvantage is that cationic lipid/DNA complexes tend to show a discrepancy between in vitro and in vivo transfection capabilities and thus there is a need for specific tailoring for each in vivo trial and route of administration.

Successful clinical and pre-clinical trials using lipoplexes have been performed. For example, clinical trials for the treatment of cancer by the direct injection of HLA-B7 glycoprotein DNA into metastatic melanoma tumors (Nabel et al. 1993). Here a CTL response was detected in all 5 patients, one of which showed tumor regression. Other examples include aerosol mediated delivery of chloramphenicol acetyl transferase (CAT), to the lung epithelial cells of mice for at least 21 days (Stribling et al. 1992) and delivery of CFTR to the lung epithelial cells of CF patients showing a significant correction of their chloride channel abnormalities (Alton et al. 1999).
Chapter 1 Introduction

Properties of an Ideal Vector

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
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<tbody>
<tr>
<td>Easy production</td>
<td>The virus should be easy to manipulate and grow to high titre. Vectors must allow for easy manipulation of their genes and regulatory elements and subsequent insertion of transgenes. Production of high titres are necessary for transduction of the large number of cells often required for gene therapy.</td>
</tr>
<tr>
<td>Long term expression</td>
<td>Once delivered the vector should for many applications have the ability to express the transgene for a sustained period of time and if necessary in a regulated fashion.</td>
</tr>
<tr>
<td>Immunologically inert</td>
<td>The vector should be able to avoid immunological clearance, avoiding both humoral and cell mediated responses.</td>
</tr>
<tr>
<td>Wide host range</td>
<td>An Ideal vector would have a broad host range and be able to infect both dividing and non-dividing cells, although in cancer therapies it is advantageous to only infect dividing cells.</td>
</tr>
<tr>
<td>Cell targeting</td>
<td>Vector targeting to specific cell types can also be highly desirable. This is particularly the case where the target cells are distributed throughout the body or are part of a heterogeneous population.</td>
</tr>
<tr>
<td>Size capacity</td>
<td>The vector should accept inserts of varying sizes. Genes delivered in gene therapy protocols vary greatly in size, insulin being 350bp (Demeterco and Levine 2001) and Dystrophin ~14kb (Hartigan-O'Connor and Chamberlain 2000).</td>
</tr>
</tbody>
</table>

Table 1.2. Summary of some of the properties that would be possessed by an ideal gene therapy viral vector.
1.4.2. Viral Vectors.

The first deliberate use of a virus in disease therapy took place over one hundred years ago, when the cowpox virus was used for vaccination against small pox (Nolan and Shatzman 1998). Since then viruses and virus subunits have been extensively used in vaccination strategies against viral disease. Viruses are now also being developed as vectors for gene therapy as they have naturally evolved to enter cells in order to deliver their genetic material.

Perhaps the biggest blow to gene therapy to date came in September 1999 with the death of Jesse Gelsinger (Lehrman 1999). Jesse suffered from partial deficiency of the liver enzyme ornithine transcarbamylase (OTC), which is characterised by the build up of toxic levels of ammonia within the body. He was taking part in a phase 1 escalating dose clinical trial, using an E1-deleted E2A temperature sensitive adenovirus vector (see later). Jesse was one of the two patients who were receiving the highest dose of the adenoviral vector used (3.8 \( \times 10^{13} \) virus particles). Many questions have been raised since this death and some researchers have questioned why patients suffering from partial enzyme deficiency were even involved in the trial, since a strict diet and ammonia clearing compounds can control this mild form of the disease. Since this time the public perception of gene therapy has suffered a severe setback.

1.4.2.1. Retroviral Vectors.

Retroviruses are enveloped single stranded RNA viruses with genomes of 7-11kb. The genome encodes three core genes termed \( gag, \) pol and \( env \) (see figure 1.2). The coding sequences are flanked by two long terminal repeats (LTRs), and a packaging signal (reviewed in Murphy 1999; Glorioso et al. 2001). Upon entry into a host cell the genome is reverse transcribed to double stranded DNA and is then subsequently integrated into the host cell genome as a provirus (Boris-Lawrie and Temin 1994). Retrovirus integration into the host cell genome thus provides the basis for long term transgene expression. However integration is not site specific and thus this can result in activation of oncogenes or inactivation of tumor suppressor genes (Donahue et al. 1992). Despite this possibility there has been little reported evidence of this phenomenon so far (see later).
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Retroviruses have long been the vector in most widespread use for gene therapy protocols since they are relatively simple and easy to manipulate. Most retroviruses are relatively non-pathogenic and harmless in humans. Currently 40% (http://www.wiley.co.uk/genetherapy/vectors.html) of gene therapy protocols utilise retroviral vectors, most commonly those derived from Moloney murine leukemia virus, MoMuLV (Vile 1997). Vector construction involves the removal of all viral genes and their replacement with the transgene of interest, flanked by the remaining cis regulatory elements (reviewed in Murphy, 1999) (see figure 1.2.). This renders the resulting virus replication incompetent but able to integrate and direct transgene expression. This allows for the insertion of up to 8kb of foreign genetic material but thus presents upper size constraints on the therapeutic gene that can be inserted (Miller 2000). Complementing cell lines have been produced that express the gag, pol and env genes in trans to allow the production of recombinant vector (Miller 1990). Separation of the gag/pol genes from the env gene provided a cell line that had a reduced risk of generating replication competent retroviruses (RCR) through recombination, which has otherwise posed a considerable concern (see figure 1.2.). Donahue et al (Donahue et al. 1992) reported that their retroviral producer cell line generated a low concentration of RCR alongside the retroviral vector. However after ex vivo transduction of primate stem cells with a retroviral vector and re-implantation back into the rhesus monkey, three out of ten monkeys developed a T cell neoplasm. This was thought to be as a result of the RCR in the retroviral vector stock used. This work provided evidence that unintentional tumor production could occur after gene transfer and has highlighted the need to achieve RCR free retroviral vector preparations.
Figure 1.2. Schematic representation of recombinant retroviral vector production. A. The viral genome consists of the \textit{gag}, \textit{pol} and \textit{env} genes plus the packaging sequence X. B. The packaging cell line constitutively expresses the 3 viral genes, separating \textit{gag} and \textit{pol} from \textit{env} in order to reduce the chances of creating replication competent vectors. After transfection of a cassette, containing a transgene flanked by LTRs, into the cell, vector genomes are produced. Vector genomes are then packaged into capsids along with the \textit{gag} and \textit{pol} proteins and the virus buds from the cell surface. The resulting retroviral particles are then ready to infect target cells.
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Retroviruses have a broad host range that is dependent on the tropism of the envelope protein (Miller 2000). One of the major drawbacks of retroviruses is their inability to infect non-dividing cells, as they require the onset of mitosis in order to enter the nucleus (Miller et al. 1990). To overcome this problem many retroviral protocols use an ex vivo gene therapy approach, as certain cells such as stem cells can be stimulated to divide in vitro (Salvatori et al. 1993 and reviewed in Verma and Somia 1997). Additionally, efforts are underway to add receptor specific ligands or alter retroviral envelope glycoproteins, (pseudotyping), in order to modify the virus tropism (Cosset and Russell 1996; Palu et al. 2000). Recently a MMLV retroviral vector pseudotyped with vesicular stomatitis virus G protein (VSV-G) was used to create a transgenic male rhesus monkey expressing GFP (Chan et al. 2001). The aim of the work was to overcome the problems in traditional gene transfer methodology used to produce transgenic primates. Here the vector, containing GFP driven by the elongation factor alpha-1 promoter (EFalpha-1P), was injected into mature rhesus oocytes and six hours later fertilised by direct sperm injection. Out of 224 fertilised oocytes, three live births were achieved. Of these one male contained the transgene in all tissues analysed. This was the first demonstration that a transgenic primate could be produced.

1.4.2.2. Lentiviral Vectors.

Lentiviruses are a subgroup of the retrovirus family, the best known members being human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). The cell surface receptor for lentiviruses is CD4 and thus they infect macrophages and lymphocytes (Naldini 1998). A major advantage of the lentivirus family for vector use is that they have evolved to infect non-dividing cells (Naldini 1998). This phenomenon has been attributed both to a nuclear localisation signal in the preintegration complex and the gag matrix protein which contains a nuclear localisation sequence. This directs nuclear import upon conjugation with another unknown protein (Bukrinsky et al. 1993). Lentiviral vectors based on HIV were one of the first lentiviral vectors to be pseudotyped. This meant that their host range could be expanded beyond CD4+cells. The viral envelope protein was substituted for the vesicular stomatitis virus G protein (Burns et al. 1993; Naldini et al. 1996), thus facilitating
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virus entry into a wide range of cells including neurons, muscle fibres and hepatocytes. Pseudotyping allowed some of the first real demonstrations of the practical use of lentiviral vectors. However, these first generation HIV-based vectors gave rise to concerns over the possibility of creating infectious HIV particles through recombination. Since then lentiviral vectors with further gene deletions have been developed (see later).

The lentiviral genome is more complex than other retroviral genomes, in addition to gag, pol and env, HIV also contains two regulatory genes, tat and rev and four accessory genes, vif, vpr, vpu and nef (Trono 1995). The four accessory genes appear to have no obvious function in vitro, but are required for full virulence in vivo and thus do not need to be complemented in culture (Naldini 1998). Vectors have been constructed that have deletions of all four accessory genes which give effective transduction of both dividing and non-dividing cells both in vivo and in vitro (Kim et al. 1998;Zufferey et al. 1997).

Additionally a third generation of lentiviral vectors requiring only gag, pol, rev and VSVG have now been produced, which do not encode rev or tat (Dull et al. 1998). Other significant advances in lentiviral vector safety have been achieved by the generation of self-inactivating viruses (Delviks et al. 1997;Miyoshi et al. 1998). Miyoshi et al used a transfer construct that had almost a complete deletion in the 3' LTR including the TATA box and binding sites for transcription factors. This deletion is transferred to the 5' LTR during reverse transcription, the deleted LTRs becoming integrated into transduced cells. The integrated provirus is therefore non-functional due to the loss of its cis-acting elements in the LTRs. Inserted promoters in the transgene cassette drive the gene expression.

1.4.2.3. Adenoviral Vectors.

Adenoviruses (Ads) are non-enveloped double stranded DNA viruses with genomes of approximately 36kb in length, which usually remain episomal elements within the host cell nucleus. In humans there are 50 different serotypes, which are classified into six subgroups termed A-F (reviewed in Horwitz 1996). Members of the subgroup C are generally thought to be the most suitable for gene therapy vectors, due to their low pathogenicity and of this
subgroup, Ad1, Ad2, Ad5 and Ad6 are regarded as endemic within the population (Murphy 1999). The genome is functionally divided into two major regions, termed late and early, according to their time of transcription after infection. These two regions contain overlapping transcriptional units and in total code for over 50 polypeptides (reviewed in Shenk 1996), see figure 1.3.

![Adenovirus Genome Diagram](image)

**Figure 1.3. Schematic representation of the adenovirus genome.** The black arrows show the four early coding regions, of which E1A and E3 are transcribed from the upper DNA strand and E3 and E4 are transcribed from the lower strand. The blue arrows show the late coding regions, L1-5. The genome is flanked by the cis-acting inverted terminal repeats (ITRs) and the packaging sequence \( \psi \).

The tropism of Ads and their resulting vectors is extremely broad and they can infect both dividing and non-dividing cells (Benihoud *et al.* 1999). The primary cell targets in natural infection are epithelial cells of the respiratory tract. After infection the E1A gene is the first gene to be transcribed, and its products act to transactivate the expression of the other early and late genes (Shenk 1996). The E3 gene product is non-essential *in vitro* but *in vivo* it plays an important role in the persistence of adenovirus infection as it acts to inhibit a cytotoxic T-lymphocyte (CTL) induced lysis of the adenovirally infected cell (Beier *et al.* 1994). First generation Ad vectors were deleted for the E1 region so that theoretically the virus would be unable to replicate on non-complementing cell lines, due to its inability to transactivate other early genes. The human embryonic kidney cell line 293 was produced that constitutively expressed the E1 gene products in order to allow the *in vitro* growth of first generation Ad vectors (Graham *et al.* 1977). Generally these vectors also had the E3 coding
region deleted, since it is not required in vitro, in order to increase the packaging capacity of the vectors to approximately 7.5kb (Bett et al. 1993). However it was found that these vectors still produced low levels of the remaining viral genes in vivo, probably due to a cellular E1A-like transactivating factor (Yang et al. 1994). Thus transgene expression from these vectors, with few exceptions, was found to be transient most likely due to host immune responses to the foreign viral antigens (reviewed in Murphy 1999).

Following the limited prospects of first generation adenoviral vectors, increasingly attenuated vectors have since been developed (reviewed in Glorioso et al. 2001 and Murphy 1999). Second and third generation viral vectors were created that were deleted for the E2 (Engelhardt et al. 1994; Yang et al. 1994) and E4 regulatory regions (Dedieu et al. 1997), in addition to E1 and E3, and were thus less immunogenic to the host. These vectors now tend to have a minimal 19kDa part of the E3 coding region remaining, since it is thought to be responsible for inhibition of CTL induced cell lysis (Beier et al. 1994).

Although these second and third generation vectors have decreased cytotoxic profiles, transgene expression generally remains short. Since then gutless adenoviral vectors have been produced that contain no viral genes (Kochanek et al. 1996; Parks et al. 1996). Production of these gutless vectors relies on a helper-packaging virus. The helper virus contains all of the necessary Ad replication and packaging signals that act in trans on a vector containing the necessary cis acting elements and the foreign gene. The helper virus is unable to package itself but aids the production of recombinant virions that lack all the Ad genes except for the cis acting elements. Systems of this nature allow for an increased packaging capacity of the recombinant virus, up to 36kb, and theoretically should have a reduced cytotoxic profile compared to earlier Ad vectors, due to the lack of Ad proteins. However one drawback to this system is the inability to completely remove the helper virus from recombinant vector preparations (Glorioso et al. 2001). Parks et al (Parks et al. 1996) have developed a method to produce gutless adenoviral vectors using a 293/ Cre-
recombinase packaging cell line that reduces the helper virus in vector preparations to very low levels (see figure 1.4.).

A major drawback of adenoviral vectors is the host immune response, which arises at both the cellular and humoral levels (Yang et al. 1995). It has been highlighted that the cellular immune response to Ad vectors does not require de novo viral gene expression and that even gutless vectors will not completely abrogate the host immune response (Kafri et al. 1998). In the case of gene therapy the humoral response could render the individual intolerant to further administration of Ad vector. However one potential method to circumvent this limitation would be to utilise the 50 different adenovirus serotypes for repeat administrations (Parks et al. 1999).
Figure 1.4. Schematic representation of 'gutless' adenoviral vector production using the Cre/lox system. The Ad helper virus contains two loxP sites flanking the packaging signal ψ. The Ad expression cassette contains the transgene flanked by stuffer DNA and the adenoviral cis regulatory elements. After transfection into 293 cells expressing Cre-recombinase, the helper virus provides all the necessary gene products in trans that are necessary for replication and packaging of an Ad vector containing the appropriate regulatory sequences. Cre recombinase mediates the conditional excision of the packaging signal in the helper virus such that it cannot be incorporated into the new virion particles. Thus there is minimal contamination of recombinant Ad particles with helper virus.
1.4.2.4. Adeno-Associated Virus.

Adeno-associated virus (AAV) belongs to the parvovirus family and is a single stranded non-pathogenic DNA virus. In humans there are 6 serotypes of which most gene therapy studies focus on the best-characterised serotype AAV-2 (Murphy 1999). The non-pathogenic nature of these viruses makes them potentially powerful tools for gene therapy protocols (reviewed in Rabinowitz and Samulski 1998). AAV requires the presence of a helper virus, usually adenovirus or herpes simplex virus, in order to establish a productive infection. In the absence of helper virus AAV integrates into the host cell genome (Berns et al. 1975). Wild type AAV preferentially integrates into a specific locus in chromosome 19, facilitated by the rep gene products (Muzyczka 1992). However AAV can also transduce cells without integration (Duan et al. 1999). AAV infects a wide variety of cell types that are both dividing and non-dividing (Samulski 1998), another benefit for gene therapy protocols.

The AAV-2 genome is 4680bp and consists of a packaging signal and ITRs flanking two coding regions, rep and cap. The rep gene encodes the proteins involved in replication and integration and the cap gene codes for capsid proteins (reviewed in Verma and Somia 1997).

Creation of AAV vectors usually involves the replacement of the rep and cap genes with a transgene. The loss of the rep gene means that recombinant AAV vectors loose their advantage of preferential integration into chromosome 19 and AAV vectors are thought to integrate at random sites (Muzyczka 1992). AAV vector propagation thus requires complementation of the rep and cap genes plus additional complementation with a helper virus. To produce AAV vectors, usually the AAV plasmid, (containing the therapeutic gene, ITRs and packaging signal) is transfected with a plasmid containing the rep and cap genes and either a helper virus or helper virus genes (either an attenuated helper virus in e.g. 293 cells, or by using a third plasmid, containing essential virus genes) (Grimm and Kleinschmidt 1999). The advantage of using a third plasmid is that it reduces the risk of the helper virus contaminating the rAAV stocks, but here there are considerable problems in generating high titres of AAV vector that are only recently being addressed (Xiao et al. 1998).
toxicity associated with the required *rep* and helper virus proteins has thus far made the generation of packaging cell lines very difficult (Verma and Somia 1997). An alternative approach made by Conway *et al*, (Conway *et al*. 1999) used a recombinant replication defective herpes simplex virus (HSV) containing the AAV *replcap* genes. When used to infect a cell line containing the AAV vector genome, the HSV vector supplies both helper virus and AAV genes in a single simple step, which avoided transfection. Since transfection is hard to scale up, this potentially provides a significant advance in the area.

The small size of the AAV genome means that the vector has a limited packaging capacity of 4.7kb (Kremer and Perricaudet 1995), including ITRs and regulatory sequences, a major disadvantage of this vector system. However some groups have recently made significant advances in overcoming this problem (Nakai *et al*. 2000; Sun *et al*. 2000; Yan *et al*. 2000) utilising the fact that AAV genomes usually concatermerise after transduction. Based on this ‘concatermerisation a candidate gene can be split in two and each half placed separately into two AAV vectors. Upon transduction the relevant 5’ and 3’ ITR’s can fuse and after mRNA processing resulting from appropriately positioned splice donor and acceptor sites, a functional full length gene is produced. Nakai *et al* (Nakai *et al*. 2000) split an expression cassette such that one vector contained a promoter-less nuclear localising LacZ gene (nlsLacZ), and another contained the human EFalpha-1 promoter/enhancer (EFalpha-1EP). After injection of the two vectors into the portal vein of a mouse, LacZ expression was observed at levels of about 60-70% of that obtained using a vector containing a complete nlsLacZ/EFalpha-1EP expression cassette. This provided evidence that AAV concatermerisation indeed occurs and that it can facilitate the fusion of split genes.

1.4.2.5. Other Viral Vectors.
Most current gene therapy protocols are concerned with the viral vector systems utilising retroviruses, lentiviruses, adenoviruses, AAV or HSV (see later). However there are now other viral systems which have demonstrated a growing potential. One such system is a hybrid vector that encompasses attractive features of more than one virus. Leiber *et al* (Lieber *et al*. 1999)
produced an adenovirus-AAV hybrid vector that aimed to couple the advantages of Ad (high titre, high infectivity and large insertional capacity) with the long-term gene delivery and integration capability seen with AAV vectors. Using Ads it had previously been shown (Steinwaerder et al. 1999) that inverted repeat sequences (IRs) inserted into an Ad vector genome mediated predictable genomic rearrangements resulting in vector genomes devoid of all viral genes. Such genomes termed DeltaAd.IR, were packaged into functional Ad particles and contained only the transgene cassette flanked by repeat sequences, packaging signal and ITR sequences. It was subsequently demonstrated that a hybrid vector, DeltaAd.AAV, could also be produced as a by-product of first generation Ad-AAV vector amplification (Lieber et al. 1999). DeltaAd.AAV genomes contained only the transgene flanked by AAV ITRs, Ad packaging signals and Ad ITRs. These vectors could be produced at high titre and purity, were thought to integrate into the host cell genome in vitro and had a reduced cytotoxic profile compared to first generation Ad vectors. They thus presented a promising tool for further gene therapy protocols.

Additionally, hybrid vectors using HSV and AAV (see previous) (Conway et al. 1999) and Ad and retroviruses have also been reported (Feng et al. 1997; Zheng et al. 2000). Zheng et al produced a replication defective Ad vector containing MMLV LTRs flanking the luciferase reporter gene. They showed in vivo gene expression for up to six months and vector integration into the host cell genome of both dividing and non-diving cells.

Other viral vector systems studied include Epstein-Barr virus (EBV) (Sclimenti and Calos 1998), SV-40 (Strayer 1999) and alpha-viruses (Wahlfors et al. 2000). EBV, a human herpes virus, contains two important components relevant to vector use, the latent origin of replication, (oriP), and the Epstein-Barr nuclear antigen (EBNA-1). These elements have the ability to confer replication and retention of genomes as extra-chromosomal units during cell division. An HSV-1 amplicon (see later) based hybrid vector containing oriP and EBNA-1 has been demonstrated to maintain the hybrid vector extra-chromosomally for six months (Wang and Vos 1996).
1.4.3. **Herpes Simplex Virus.**

1.4.3.1. **A Vector for Gene Therapy.**

Herpes simplex virus is a member of the alpha herpes virus family (Roizman et al. 1981). The two members of the HSV group, HSV-1 and HSV-2 are responsible for oral and genital herpes respectively. HSV-1 has been the most intensively studied to date and possesses a number of important features that make it potentially an ideal vector for gene therapy in certain tissues (reviewed in Fink et al. 1996 and Coffin and Latchman 1996). Such features include: the natural infection of both dividing and non-dividing cells; a life cycle which includes a period of latency, potentially allowing long-term transgene expression; a large genome which allows the insertion of large pieces of foreign genetic material; relatively straightforward manipulation of the genome and high titre viral growth. These properties provide a significant rationale for the development of HSV-1 vectors for gene therapy.

1.4.3.2. **Biology of HSV-1.**

HSV-1 is a double stranded DNA virus with a genome of 152kb coding for over 80 polypeptides (Roizman and Sears 1996). It comprises two unique regions, short and long, each of which is flanked by inverted terminal repeats (see figure 1.5.). The unique long and unique short regions of the genome can invert relative to one another, thus yielding four possible linear isoforms during replication (Jacob et al. 1979). Approximately half of the 80 proteins encoded by HSV are essential for virus replication in vitro, but the rest are dispensable. Many of these dispensable genes are however necessary for full pathogenesis in the host (see later).

The natural route of HSV-1 infection involves uptake of the virus by epithelial cells of the skin, where-upon the virus undergoes lytic gene expression and replication (reviewed in Roizman and Sears 1996). Following lytic replication the virus infects sensory nerve terminals and is transported retrogradely to the nerve cell body. The virus can then either undergo a further round of lytic replication or the genome can remain an episomal nuclear element and enter a period of latency. Sensory neurons are the natural site of herpes latency and
as such the virus is thought of as neurotrophic. However *in vitro* and *in vivo* HSV-1 has been shown to infect a broad range of cell types. Because HSV-1 has naturally evolved to infect neurons as part of its lifecycle, unlike other viruses used as vectors, this has made it an attractive vector for development of gene therapy in the nervous system. Additionally the unique property of retrograde transport gives the vector an advantage over other virus systems, in that it can be administered peripherally followed by transport to the host cell nucleus, rather than the need for direct injection at the target site.

The HSV-1 virion consists of an icosahedral capsid containing the DNA genome, surrounded by a membranous envelope. Separating the envelope from the capsid is a protein filled tegument (Roizmans and Sears 1996). The tegument contains virion proteins, most notably virion protein 16 (VP16 alternatively known as, α-tif, vmw65 and ICP25) (Spear and Kellejmoian 1970) and the virion host shut-off protein (vhs) (Kwong et al. 1988). The envelope contains at least 11 glycoproteins, from glycoprotein B (gB) through to glycoprotein (gM), which facilitate virus attachment and entry into the cell (reviewed in Roizman and Sears 1996; Fink et al. 1996).

There is still debate over which glycoproteins are responsible for cell attachment, but the mechanism is likely to involve more than one pathway and more then one glycoprotein. This is supported by the fact that HSV must attach to and penetrate different types of cell membrane during its lifecycle, including those found on epithelial cells and neurons. Evidence for the involvement of gB, gC and gD has been observed (Fink et al. 1996). Heparan sulfate is the best characterised cell surface receptor for the virus (Shieh and Spear 1991; WuDunn and Spear 1989), but is likely to function alongside other surface receptors. Another possible receptor is fibroblast growth factor receptor (FGFR) (Baird et al. 1990; Kaner et al. 1990). After membrane attachment, HSV-1 entry results from the fusion between viral envelope and the cell membrane, rather than by phagocytosis (Morgan et al. 1968). Again this is a multi-step process that involves more than one glycoprotein, most notably gB, gD, gH and gL (Fink et al. 1996). Upon entry into the cell the capsids are released into the cytoplasm and transported to the nucleus (Batterson et al. 1983). Fusion of the membrane
also allows the release of the tegument proteins vhs and VP16. Vhs remains in the cytoplasm and is responsible for shutting-off host protein synthesis, by the non-selective degradation of mRNA (Kwong et al. 1988; Oroskar and Read 1989). VP16 is transported to the nucleus whereupon it transactivates the promoters of the immediate early genes to initiate the lytic gene expression cascade (Campbell et al. 1984).

![Schematic diagram of the HSV-1 genome](image)

**Figure 1.5. Schematic diagram of the HSV-1 genome.** Unique long (UL), and unique short (US), regions are flanked by the terminal and internal short and long repeat regions (TRS, IRS, TRL, TRS). The genome has three origins of replication one in the unique long region, OriL, and two copies of OriS in the short repeat region. The 'a' sequences in the repeat region contain the signals required for cleavage and packaging of viral DNA.

1.4.3.3. Gene Expression.

During lytic replication the HSV-1 genes are transcribed in a temporal cascade and are classified according to their immediate early (IE or α), early (E or β), or late (L or γ), time of expression after entry into the host cell (Hones and Roizman 1974). There are five IE gene products, infected cell proteins (ICP)0, ICP4, ICP22, ICP27, and ICP47. With the exception of ICP47 all the IE proteins regulate the expression of the remaining HSV-1 genes.

ICP47 functions to inhibit the transporters of antigen processing (TAP), which are responsible for peptide transport across the ER membrane, thus reducing the presentation of HSV antigens on the surface of infected cells by MHC class 1 (York et al. 1994). It has been demonstrated that the ICP47 protein binds to
the cytosolic portion of the TAP heterodimer and thereby prevents peptide translocation across the ER membrane (Fruh et al. 1995; Hill et al. 1995). This results in empty MHC class I molecules in the ER. It was suggested that the ability of HSV-1 to enter latency, with periodic reactivation and lytic replication, without undergoing immunological clearance, is a function of selective avoidance of the host’s CD8+ T-cell response. Our laboratory has found that the vhs protein is responsible for preventing dendritic cells becoming activated following infection, a second method by which HSV probably avoids the generation of cellular immune responses and thus clearance (Laila Samedy, unpublished observations).

Transcription of the IE genes by VP16 requires interaction with the cellular factors Oct1 and Host Cell factor (HCF). These bind to the 5’TAATGARAT3’ (where R is purine) sequences contained in the regulatory regions of each of the IE genes (Gaffney et al. 1985), following which transactivation occurs. Oct-1 is part of a family of homeobox proteins that share a structure known as the POU domain (Herr et al. 1988). The bipartite POU domain is responsible for the recognition and binding to a number of octamer based sequences, such as that found in the HSV-1 IE genes (Herr and Cleary 1995). HCF (or C1) is thought to play an important role in transporting VP16 to the cell nucleus (La Boissiere et al. 1999). The VP16 protein does not interact with DNA directly but forms a complex with HCF, which then binds to the Oct-1/TAATGARAT unit (Preston 2000). This functions to bring the C-terminal domain of VP16 into close proximity of the pre-initiation complex on the TATA box (TFIID, TFIIB etc.) ready to begin trans-activation. VP16 is itself a late gene product and in addition to its role in IE transactivation it is an essential structural component of the tegument. The essential structural function of VP16 is contained within the N terminal domain and has been shown to be absolutely required for productive HSV infection (Poon and Roizman 1995; Weinheimer et al. 1992).
Figure 1.6. The HSV-1 replication cycle. VP16 and VHS (virion protein 16 and virion host shut-off protein) are virion proteins and after fusion of the cell membrane with the viral envelope they enter the cell along with other virion proteins. Immediate-early (IE) gene expression is initiated by transactivation of their promoters by VP16 and thus the temporal cascade of gene expression and lytic replication begins. ICP6 expression is independent of IE transactivation.
1.4.3.4. The Immediate-Early Genes.
The four transactivating IE genes, ICPO, ICP4, ICP22 and ICP27 all play slightly different roles in the viral replication cycle. ICPO is capable of transactivating all three classes of viral genes, IE, E and L. Whilst it is not essential for viral growth, mutants lacking this protein demonstrate significantly reduced levels of gene expression and viral yields, especially at low multiplicities of infection (MOI) (Cai and Schaffer 1992; Chen and Silverstein 1992; Stow and Stow 1986). At high MOI mutants display a nearly wild-type phenotype. It has been suggested that the activation of gene expression by ICPO occurs at the level of transcription (Jordan and Schaffer 1997).

There are two copies of the gene encoding ICPO as it is present in the long repeat regions of the HSV-1 genome. The RNA is in part antisense to the latency associated transcripts, LATs (see later). ICPO is considered a promiscuous transactivator for HSV and non-HSV genes and has been shown in transient expression systems to turn on the expression of otherwise silent genes (Everett 1985; Mosca et al. 1987; Nabel et al. 1988; Samaniego et al. 1998; Roizman and Sears 1996). By using an attenuated virus where IE gene expression was limited to ICPO and negligible levels of the remaining IE genes (d106), Hobbs and Delucca (1999) were able to show that a further consequence of ICPO expression is the induction of cell cycle arrest. This cell cycle arrest was not seen using a further attenuated virus that expressed none of the IE genes (d109). The effect of cell cycle inhibition leads to cell death and is thus toxic to the cell. However, as highlighted in their findings, it is possible that ICPO does not act alone, although it is necessary for, the cell cycle arrest observed.

ICP4 is an essential IE protein (DeLuca et al. 1985), for which the gene is found in the short repeat regions of the HSV-1 genome. ICP4 is essential for virus growth and functions to both repress IE gene expression, including an autoregulatory role (Michael and Roizman 1993; Roberts et al. 1988), and to positively regulate the expression of E and L genes (DeLuca and Schaffer 1985a). ICP4 is thought to act as a homodimer (Shepard et al. 1990) and binds directly to DNA at consensus and non-consensus sites. It mediates its function
via contact with cellular transcription factors such as the TATA binding protein and TFIID (Smith et al. 1993).

ICP27 is an essential IE gene (McMahan and Schaffer 1990; Sacks et al. 1985) which functions as both an activator and repressor of transcription. ICP27 is required for the move from early to late gene expression and functions to repress early and activate late gene expression (McCarthy et al. 1989; Rice and Knipe 1990). ICP27 is known to act posttranscriptionally (Smith et al. 1992) and inhibits pre-mRNA splicing in the cell, providing an advantage for the virus, the RNA of which is predominately unspliced (Hardy and Sandri-Goldin 1994; Sandri-Goldin and Mendoza 1992). ICP27 also negatively modulates the expression of the IE genes ICP0 and ICP4 (McMahan and Schaffer 1990; Rice and Knipe 1988).

ICP22 is dispensable for virus growth in many cell types but acts to promote late gene synthesis in a cell type dependent manner (Sears et al. 1985). It also been implicated in the production of an aberrantly phosphorylated form of cellular RNA polymerase II (Rice et al. 1995).

The manner in which the IE genes interact to regulate the expression of other HSV-1 proteins is complicated and not yet fully understood. However, the importance of their functions can clearly be seen. Following the expression of IE genes the E genes are expressed. These are mainly responsible for viral DNA synthesis. Early gene products include thymidine kinase (tk), ICP8 (the major DNA binding protein) and DNA polymerase. Late gene synthesis is only triggered after viral DNA synthesis has occurred. Late gene products include ICP34.5 (the neurovirulence factor) and structural proteins such as the glycoproteins. ICP6, the large subunit of ribonuclease reductase, is unusual because it is classified as a hybrid IE/E gene. It was discovered that ICP6 expression is independent of IE transactivation and is expressed at a delayed IE time (Sze and Herman 1992).

Viral DNA synthesis occurs by a rolling circle mechanism that produces head to tail concatamers of the HSV-1 genome, during which the unique short and long regions can invert relative to one another. The viral DNA is then cleaved into
genomic lengths and packaged into capsids through the recognition of the "a" packaging sequences found in the repeat regions. The capsids then acquire the tegument proteins, bud through the nuclear envelope, and appropriate the viral glycoproteins and are then released by the lysing cell (reviewed in Roizman and Sears, 1996)

1.4.3.5. Herpes Simplex Virus Latency.
Herpes Simplex Viruses 1 and 2 are characterised by their ability to latently infect the sensory nerve cell nucleus. This period of latency can occur for long and even life-long periods of time, during which periodic appearances of lytic infection can occur in epithelial cells at the site of the initial infection. The signals received and mechanisms whereby the virus reactivates and switches from lytic gene expression to the latent life cycle is a contentious issue but remains a highly active area of research.

' During latency the virus genome is thought to remain as a circular episomal element in the host cell nucleus. During this period all lytic gene expression is silenced, and no viral particles or viral proteins can be detected. The only area in the HSV-1 genome that remains transcriptionally active is located in the long repeat region, and produces a family of RNAs known as the latency associated transcripts, LATs (Rock et al. 1987; Spivack and Fraser 1987; Stevens et al. 1987). The major most abundant LAT is a 2kb stable non-polyadenylated species (Farrell et al. 1991), although other less abundant LATs of 1.5kb and 1.45kb are also easily detected in neurons. These LATs are thought to be introns spliced from a larger species (Dobson et al. 1989). This primary LAT is an 8.3kb polyadenylated transcript present in very low copy number in latently infected neurons (Zwaagstra et al. 1990). This RNA initiates downstream of a TATA containing promoter element (LAP1) and extends 8.3kb downstream to the nearest polyadenylation site. The positions of the LATs mean that they run antisense, thus complementary, to the ICP0 gene. The large transcript runs entirely antisense to the gene whereas the 2kb and 1.5kb species overlap only the 3' end of the gene (see figure 1.7).
1.4.3.6. The Latency Associated Promoters.

Several studies have set out to determine how LAT expression is regulated. The LAT region contains two promoter elements, termed latency associated promoters 1 and 2 (LAP1 and LAP2 respectively) (Dobson et al. 1989; Goins et al. 1994). Dobson et al. provided the first evidence of the LAP1 promoter observing that a TATA box containing element was located almost 700bp upstream of the 5' end of the 2kb LAT coding region. This region also contained Sp1 sites, CAAT box homology and putative cyclic AMP response elements. Dobson et al. created a recombinant virus containing a β-globin gene inserted 17bp downstream of the TATA box. With this virus they found in the mouse PNS that β-globin specific mRNA was detected but no LAT. Additionally, in a similar experiment, a virus containing no TATA box was shown to express no LAT during latent infection.

Goins et al. (Goins et al. 1994) demonstrated the presence of an additional promoter element downstream of LAP1, named LAP2. The authors performed transient transfection assays using the chloramphenicol acetyltransferase (CAT) reporter gene juxtaposed to the putative promoter (the region between LAP1 and the 5' end of the major 2kb LAT). They identified a 600bp region, calling it LAP2, which lacked a TATA box but had promoter activity. They reported that the LAP2 promoter was also capable of driving low level lacZ expression in latently infected mouse trigeminal ganglia for up to 300 days pi (when inserted into the gC locus of a recombinant HSV-1 vector), although this data has never been shown (Goins et al. 1994).
Figure 1.7. The LAT region of the HSV-1 genome. The internal long repeat region is shown although there is the same arrangement in the terminal repeat region. The primary 8.3kb LAT is spliced to produce smaller LATs of 2kb and 1.5kb, which are abundant during latency. The ICP0 immediate early gene transcript is shown and is antisense and thus complementary to the LATs. The two latency associated promoters, LAP1 (containing a TATA box) and LAP2 are shown. Open reading frames, OrfO and OrfP, are also shown and are antisense to ICP34.5, the neurovirulence factor.
1.4.3.7. LAT Function.
The function of the latency associated transcripts and the mechanism whereby herpes simplex virus moves from lytic gene expression to latency (and the reverse) remains a topic of continuing and exciting research. Since the LATs are the only transcripts that can be reliably detected during latency, it has been speculated that they are likely to function in either the establishment of, or the reactivation from latency. These ideas will be discussed.

Subsequent to the discovery that the 2kb LAT was probably a stable intron (Farrell et al. 1991) a conceivable model was proposed whereby this major LAT functions as an antisense inhibitor of ICP0 and thus blocks reactivation from latency, or aids establishment of latency. In this model it was suggested that LAT could function to sequester ICP0 transcripts to the nucleus, or could target a LAT/ICP0 RNA complex for degradation by double stranded ribo-nucleases, thus in both cases blocking ICP0 translation. In agreement with this, a suggestion was proposed whereby LAT acts at an antisense level functioning to block IE gene expression and thus establishment of a latent infection (Thompson and Sawtell 1997). Here it was shown that LAT mutants failed to establish latency at wild-type levels and were impaired for reactivation for this reason. However these results are contrary to many earlier reports that indicate that LAT mutants establish latency at wild-type levels but have reduced reactivation efficiency. These will be discussed.

LAT transcription negative mutants have been shown to reactivate poorly by explant or by induced reactivation in the mouse (Leib et al. 1989; Steiner et al. 1990), by induced reactivation in the rabbit (Hill et al. 1990; Trousdale et al. 1991) and spontaneous reactivation in the rabbit (Perng et al. 1994). In an effort to further define the functional elements of the LAT region responsible for the reduced reactivation kinetics, several groups mapped regions within the first 1.5kb of the primary LAT transcript as being important (Bloom et al. 1996; Drolet et al. 1999; Perng et al. 1996). The fact that this 1.5kb identified region of LAT does not overlap ICP0 has led to suggestions that the main function of LAT is not an antisense effect on ICP0. A more recent report again supported the theory that LAT enhances the establishment of virus latency and thus maybe
the mechanism whereby it enhances reactivation (Perng et al. 2000). However since the same authors identified the 1.5kb LAT fragment previously reported, this weakened the argument for an antisense mechanism as proposed previously (Thompson and Sawtell 1997).

It is known that there are open reading frames (ORFs) in the LAT region. The largest of these ORFs, lying within the 2kb major LAT, is highly conserved between HSV-1 strains leading to the possibility that it encodes a protein, which functions in the establishment, maintenance or reactivation from latency (Coffin et al. 1998; Thomas et al. 1999a). Thomas et al found that deregulated expression of this LAT ORF could functionally substitute for ICP0 expression and enhance virus growth. This suggested that the regulated expression of the LAT ORF is important in regulating maintenance/reactivation from latency. The fact that bovine herpes virus 1 produces a LAT-encoded protein throughout latency (Hossain et al. 1995; Jiang et al. 1998) lends support to this hypothesis. Conversely, the fact that deletion of LAT ORFs leads to no detectable phenotype (Farreid and Spivack 1994), that the 2kb LAT accumulates and is maintained in the nucleus and that no protein has reliably been detected in vivo has meant this hypothesis is still controversial.

One final function of the LAT region is the possibility that it provides a functionally active region of the genome from which lytic gene transcription can begin. The LAT region of HSV-1 and other alphaherpesviruses has an unusual dinucleotide content and a high G+C ratio (Coffin et al. 1995). Here it was suggested that this feature allows the LAT region to remain transcriptionally active during latency, by taking up an altered DNA structure, as compared to the remainder of the HSV genome during latency.

From the evidence presented it is clear that the LAT region is a highly complex area. Whether the LATs act to i) enhance the establishment of latency ii) facilitate the reactivation from latency or iii) aid the maintenance of latency, is unclear and indeed the LATs may function at any or all of these times. The mechanisms by which they achieve any of the proposed functions is also unclear. However what seems most likely is that that the LAT region functions
by multiple mechanisms, including having an antisense effect, expressing a protein and keeping the chromatin 'open' during latency such that reactivation is ultimately possible.

1.4.3.8. The Detection of Latent HSV.
Detection of either latent viral genomes or LATs are two obvious ways by which to detect latent HSV in a neuron. Indeed the quantification of the amount of latent virus is a means by which we can study the viral and cellular genes required for the establishment, maintenance and reactivation from latency.

LATs were first detected by in situ hybridisation (ISH) techniques both in mouse and human trigeminal ganglia (Mitchell et al. 1990; Stevens et al. 1987; Croen et al. 1987). Since then a number of methods have been employed to detect HSV-1 in latently infected neurons and it has become apparent that the detection of HSV-1 LATs does not directly correlate with the presence of viral DNA. Ramakrishnan et al performed in situ PCR (ISPCR), to detect HSV-1 genomes, and in situ hybridisation, to detect LATs (using a LAT specific riboprobe), both in the rat hippocampus and trigeminal ganglia (Ramakrishnan et al. 1994a; Ramakrishnan et al. 1994b). They determined that although LATs are present in high copy number in many cells, the cells with LATs detectable by in situ hybridisation only represent a small proportion of those ganglionic neurons that contain HSV-1 genomes. This work was further supported by results in mouse trigeminal ganglia (Mehta et al. 1995) in situ PCR for HSV-1 genomes (using primers for UL30) and in situ hybridisation for LAT RNA were performed on sections of trigeminal ganglia of latently infected mice. It was found that approximately 4.8% of the 5000 neurons observed were positive for HSV-1 DNA whereas only 1.8% of 8000 neurons studied were positive for LAT RNA. However in their study, as was pointed out, detection of a single copy gene is only 50% reliable (using in situ PCR to detect all-l, the single copy mouse gene), so the actual number of neurons harbouring HSV DNA could be as high as 10%. The same group further suggested that mutants unable to synthesise or accumulate the 2kb LAT could be detected reliably by in situ DNA PCR, thus providing a means to study their phenotype (Maggioncalda et al. 1996). Since these findings further advances have been made in the detection of latent HSV-
1. A strategy employing *in situ* reverse transcription PCR (ISRTPCR) was employed to detect LATs. It was found that substantially more LAT positive cells were identified through ISRTPCR than through *in situ* hybridisation and that the number of positive cells was more similar to the number of cells positive for the HSV genome. A further approach was developed to quantify latent HSV-1 genomes at the single cell level, called contextual analysis (CXA) (Sawtell 1997). The author concluded that viral genome copies within latently infected neurons varied from <10 to >1000, and that there is a correlation between virus impot titre and both the number of neurons where latency is established and the number of viral genome copies per cell.

Collectively, these results suggest a number of interesting possibilities. LATs may be produced in varying numbers between different neuronal cells. This may be due in part to the number of genomes harboured by each of the cells, a high copy number giving rise to high levels of LATs, or to an as yet undefined 'cell product that transactivates or represses LAT expression. The transactivator could stimulate LAT expression in a limited subset of neurons, even from a single viral promoter element, or the repressor reduces expression even when high copy number of viral genomes are present. Conversely the availability of these factors could fluctuate in the neuron population as a whole, such that LAT expression is dynamic.
1.4.4. Herpes Simplex Virus as a Vector for Gene Therapy.

There are two types of HSV vector that are currently being developed, defective vectors and disabled vectors. Both types of vector attempt to harness the potential advantages of the parent virus, such as the broad host range, the large insertional capacity for foreign genetic material, the infection of post-mitotic cells, and the ease of manipulation. However, each system also has potential advantages and disadvantages.

1.4.4.1. Disabled HSV-1 Vectors.

Disabled vectors are based on attenuated virus backbones that are deleted in either or both essential and non-essential genes, reducing pathogenicity and toxicity, and as such often require a complementing cell line in order to grow. Essential genes are classified as those that are necessary for virus propagation and growth in culture. Such genes include those that code for the IE proteins ICP4 and ICP27 (Sacks et al. 1985; DeLuca and Schaffer 1985b), the structural genes (mostly of the L class that comprise the virion particle), and genes involved in DNA replication. Non-essential or accessory genes contribute to the ability of the virus to effectively replicate in spread in vivo and as such are not essential for growth in culture. The deletion/inactivation of essential genes requires that they be complemented in culture, whereas accessory genes do not require complementation (reviewed in Fink et al. 1996; Coffin and Latchman 1996). Creation of recombinant viruses is a relatively simple procedure. It involves the use of shuttle plasmids that contain a transgene of interest, flanked by HSV-1 DNA. The shuttle plasmid and HSV-1 viral DNA are transfected into permissive cells and recombination takes place. This recombination involves the insertion of the transgene, either a reporter gene or a therapeutic gene, into the HSV-1 genome at the site corresponding to the flanking DNA in the plasmid (see figure 1.8).
Figure 1.8. Schematic diagram to show the generation of disabled HSV vectors. HSV DNA is co-transfected into a permissive cell line together with a plasmid containing the transgene which is inserted into HSV DNA flanking the site of insertion. A complementing cell line is used if the viral DNA is deleted for essential genes (see text). The viral lytic cycle is initiated and recombination occurs. Recombined and replicated DNA is packaged into new viral particles and those containing the transgene can be plaque purified to a homogenous stock.
The key to eliminating vector toxicity is the prevention of viral gene expression and replication. Latent viral genomes are not toxic, since during latency lytic viral gene expression is silenced, even with a wild type virus. However, upon initial inoculation, either in the periphery or by direct CNS injection, viral gene expression may be toxic. Initial HSV-1 vectors were deleted for the non-essential gene thymidine kinase (tk), with insertions of either lacZ (Ho and Mocarski 1988) or Hypoxanthine-guanine phosphoribosyltransferase (HRPT) (Palella et al. 1988). These viruses are attenuated for growth compared to wild type virus and whilst they showed the potential of HSV as a delivery vehicle and were less toxic to neurons than wild type virus, they also highlighted that such vectors were highly pathogenic when used at high moi (Palella et al. 1989). Transgene expression from these viruses was transient, probably as a result of both promoter silencing and elimination of the virus as a consequence of the toxicity.

As HSV-1 genes are expressed in a temporal cascade it would be expected that disruptions at the IE level would block the later expression of E and L genes and thus reduce cytotoxicity. More highly disabled vectors with a deletion in the IE gene, ICP4, were reported in 1990 (Chiocca et al. 1990a;Dobson et al. 1990;Johnson et al. 1992). Results from these experiments showed that the β-galactosidase transgene was transiently expressed in the rat brain and although pathogenicity was reduced compared to wild-type virus, necrosis was still observed around the site of injection (Chiocca et al. 1990a). In the mouse PNS β-galactosidase expression was observed up to 24 weeks in sensory neurons, although in very few cells, but this showed that the virus was capable of a latent infection (Dobson et al. 1990). However an ICP4 mutant virus was shown to be highly toxic to cells in culture, including neurons (Johnson et al. 1992). It was reported that in primary fibroblasts the virus induced chromosomal aberrations and host cell DNA fragmentation. Such ICP4 mutant viruses are completely blocked for viral replication and have to be propagated in a complementing cell line. However whilst no replication occurs a subset of genes are expressed, which include the remaining IE genes, ICP0, ICP22, ICP27, ICP47, the large subunit of ribonuclease reductase, ICP6 (DeLuca et al. 1985) and OrfP (Yeh and Schaffer 1993). Thus it is likely that any toxicity observed using an ICP4
deleted virus is a result of expression of some, or all, of these genes. In addition virion proteins, such as VP16, may also contribute.

UV irradiation of virus stocks and pre-exposure of target cells with alpha interferon both result in reduced viral gene expression without damaging the integrity of the viral particle. Such treatments have been shown to markedly reduce viral toxicity supporting the idea that it is not the viral particle itself that causes toxicity, but more likely the proteins expressed (Johnson et al. 1992).

Using transient transfection assays it was reported that ICP6 and ICP47 had little effect on virus cytotoxicity (Johnson et al. 1994) and thus many studies have now looked at various combinations of IE gene deletions in order to develop a non-toxic virus. A virus deleted for ICP4, ICP22 and ICP27 (Wu et al. 1996) was shown to be much less toxic to Vero and human embryonic lung (HEL) cells than the corresponding single or double mutants. Another group created a similar virus deleted for ICP4, ICP22 and ICP27 (Krisky et al. 1998) and also found it to be similar non-toxic on Vero cells and non-toxic in primary cortical and dorsal root ganglion neurons, with cell survival up to 21 days. However Wu et al found that host cell DNA synthesis was inhibited 3 days after virus infection, resulting in impaired cell survival and attributed this to ICP0 accumulation in nuclear structures. Here ICP0 was thought to contribute to both the elevated levels of viral gene expression and transgene expression observed, confirming the role of ICP0 as a potent transactivator of promoters. A mutant virus deleted for ICP0, ICP4 and ICP27 was constructed to study the affect of IPC0 expression on cell survival and toxicity (Samaniego et al. 1997). Here it was found that deletion of ICP0 in the absence of ICP4 and ICP27 reduces toxicity and lowers the level of expression of genes from the viral genome. It was also found that at high MOI this virus was still toxic, possibly due to ICP22 expression.

Following on from this work a virus was created that was deleted/inactivated for all five IE genes in an attempt to completely abrogate viral cytotoxicity (Samaniego et al. 1998). This for the first time provided a completely non-toxic HSV vector in the cultured cells tested. The authors showed that at all moi (up
to 30) the virus appeared non-toxic to both Vero and HEL cells, in terms of cell morphology, protein synthesis and the ability to continually divide. They also showed that the virus genome was capable of long-term persistence in both HEL and Vero cells although quantification of viral genomes in Vero cells showed that by 28 days the number constituted one third of the number initially present. Consistent with previous reports the authors found that in the absence of IE proteins, transgene expression (as measured by a hCMV/GFP insertion) was completely repressed in the majority of cells directly after transfection, and no GFP expression was detected thereafter. This effect could be reversed by superinfection with an ICP0 expressing, non-GFP expressing, virus. Likewise Preston and Nicholl proposed that HSV and hCMV IE promoters were repressed in the absence of VP16, ICP4 and ICP0 (Preston and Nicholl 1997).

Other groups have taken a slightly different approach in order to produce viruses of reduced toxicity. Here VP16 is inactivated in conjunction with IE genes (Johnson et al. 1994; Preston et al. 1997; Thomas et al. 1999b; Marshall et al. 2000; Palmer et al. 2000; Lilley et al. 2001). It would seem reasonable that by removing the transactivating function of VP16, IE gene expression would drop, without the need to remove/inactivate the IE genes themselves. Since VP16 is a structural protein it cannot be deleted entirely without affecting virus assembly. However a number of reports have been published demonstrating that VP16 C-terminal mutations reduce the transactivating ability of the protein without affecting its structural role (Ace et al. 1989; Smiley and Duncan 1997).

An ICP0, ICP4 and VP16 mutant has been reported which was used to drive long-term β-galactosidase expression in the PNS (Marshall et al. 2000). The authors showed that transgene expression persisted up to 6 months after footpad inoculation and that no detectable virus replication was observed in either footpad or DRG. However since ICP4 was not deleted but was a temperature sensitive mutant (fully non-permissive temperature being 38°C), it is a highly contentious issue as to whether, after peripheral inoculation, replication is necessary for sufficient trafficking of the virus from footpad to DRG. A mouse footpad might be closer to the permissive temperature of 31°C.
than to 38°C, and thus replication might be possible, although this was discounted in the published work.

Most recently Lilley et al (Lilley et al. 2001) have created an ICP4, ICP27 and VP16 mutant. This virus contains a disabling mutation in VP16, is deleted for ICP34.5 and OrfP and deleted for ICP4 and ICP27. The authors found that on non-complementing cells this mutant exhibited minimal levels of ICP0, ICP22 and ICP47 gene expression, even at high moi, and that the only viral gene detected to significant levels was ICP6. The ICP6 protein has previously been shown to be non-toxic (Johnson et al. 1994). The same virus, with a reporter cassette inserted into the vhs or LAT locus, was shown to be non-toxic to primary DRG cultured neurons, judging by cell morphology. Additionally the virus can establish a persistent infection of Vero cells and in contrast to previous reports (Samaniego et al. 1998), can express GFP from a reporter cassette in these cells up to 23 days post infection. In the absence of IE gene expression this phenomenon was probably a direct result of incorporation of LATP2 (an extended version of LAP2) into the reporter cassette (see later). Additionally and for the first time the virus was shown to yield high levels of β-galactosidase expression in the rat striatum and substantia nigra for at least one-month post injection. This is the first report that HSV-1 vectors can support significant long-term transgene expression in the CNS and appears to be a feature attributable to the both the lack of IE gene expression (thus lack of cytotoxicity of the virus used), and the incorporation of latency associated promoters to drive transgene expression.

Although deletion of cytotoxic gene products potentially makes an ideal vector for gene therapy approaches, some situations require an agent that is replication competent. ICP34.5 is located in the long repeat region of the HSV genome and runs antisense to the 8.5kb primary LAT transcript, see figure 1.7. HSV-1 ICP34.5 mutants have been found to be non virulent in mice (Chou et al. 1990; MacLean et al. 1991a; Robertson et al. 1992; Valyi-Nagy et al. 1994). The effect of ICP34.5 gene on HSV-1 replication has been found to be tissue and cell type specific and it is generally understood that ICP34.5 mutants replicate in actively dividing, but not in terminally differentiated cells. ICP34.5 mutants are
therefore replication defective in sensory ganglia and the CNS of mice (Chou et al. 1990; MacLean et al. 1991a; Whitley et al. 1993; Valyi-Nagy et al. 1994). Replication of ICP34.5 mutants in tissue culture cells is normal in several cell types but restricted in others (Bolovan et al. 1994; Brown et al. 1994; Chou and Roizman 1992).

Currently ICP34.5 mutants are being used in phase 1 clinical trials to treat patients suffering from malignant glioma. (Markert et al. 2000; Rampling et al. 2000). These strategies are based on the assumption that after direct injection of the virus into the tumor, the virus will selectively replicate in the dividing tumor cells but will not replicate in the surrounding brain.

1.4.4.2. Defective HSV-1 Vectors.
Defective vectors, or amplicons, are based on a transgene containing plasmid, which also contains a HSV-1 origin of replication and a cleavage/packaging signal ("a" sequence) (Sena-Esteves et al. 2000; Spaete and Frenkel 1982). Amplification of the plasmid is carried out in bacterial cells and then vector propagation is traditionally carried out following tranfection into a permissive cell line followed by infection with a helper virus. Transfection and infection of the amplicon and helper virus respectively into the cell line, results in production of defective virions containing the amplicon DNA and thus the transgene of interest. The helper virus and cell line provide all the HSV-1 functions necessary for packaging of the amplicon as head to tail concatemeric units of 152kb, into virus particles. Thus within each defective virus particle there will be multiple copies of the transgene (see figure 1.9) (Fink et al. 1996; Sena-Esteves et al. 2000).

One theoretical advantage of amplicons is that there should be no restriction on the promoters chosen to drive transgene expression, as the amplicon should not be susceptible to normal silencing of the HSV promoters by the surrounding genome, which usually occurs during latency. However a significant drawback of using the amplicon system is the generation of a mixed population of helper and defective viral particles in the resulting stock. Since these do not differ in their physical properties they cannot easily be separated in vitro (Hermens and
Verhaagen 1998). This is highly problematic as \textit{in vivo} it can mean that the expression of cytotoxic HSV gene products can cause neuropathological effects. It is known that repeated passage of the mixed viral stocks on helper cells reduces the proportion of helper virus to defective virus but does not remove it (Fink \textit{et al.} 1996; Spaete and Frenkel 1982).

One of the first defective vector systems to be used was that employing the tsK virus as a helper. This has a temperature sensitive mutation in the IE gene ICP4. This virus allowed growth at 31°C, the temperature at which the defective particles were propagated, but prevented growth at the 37°C (Davison \textit{et al.} 1984). Such vectors was first used to drive \(\beta\)-galactosidase expression from the IE22/47 gene promoter in cultured rat neurons of the PNS (Geller and Breakefield 1988) and CNS (Geller and Freese 1990), plus a number of other cultured human cell types (Boothman \textit{et al.} 1989). The first \textit{in vivo} use of an amplicon grown using tsK as a helper saw the expression of \(\beta\)-galactosidase for at least two weeks in the rat brain (Kaplitt \textit{et al.} 1991). Since it is possible for the tsK mutant to revert to wild-type virus with relatively high frequency, helper viruses were subsequently employed with a complete deletion in ICP4, rather than a tsK mutation. These viruses were shown to revert to wild type with less efficiency (Geller and Freese 1990). These systems have since been modified and used to express functional genes both \textit{in vitro} and \textit{in vivo} (reviewed in Hermens and Verhaagen 1998; Coffin and Latchman 1996). Amplicon vectors have been constructed containing neuronal specific promoters such as those for preproenkephalin (Kaplitt \textit{et al.} 1994) and tyrosine hydroxylase (TH) (Song \textit{et al.} 1997). Using these viruses expression \textit{in vivo} was restricted to the appropriate cells and appeared to be more long-lived than with viral promoters.

The problems associated with reversion of helper viruses to wild type, and the production of a mixed population of helper virus and defective virus were clearly demonstrated by During \textit{et al} in 1994 (During \textit{et al.} 1994). This work reported that an amplicon virus expressing TH could induce behavioural recovery up to at least a year in a rat model of Parkinson's disease. Although they showed the potential of the amplicon system for long term therapy they also noted that 10%
of the rats died during the experiment, probably as a result of contamination of the amplicon stocks with helper virus that had reverted to wild-type.

Since 1994, helper free amplicon systems have been developed. The first system utilises a set of five overlapping bacterial cosmids that between them carry the entire HSV-1 genome, apart from the cleavage and packaging signals (See figure 1.9) (Cunningham and Davison 1993; Fraefel et al. 1996). After transfection, the cosmids provide the necessary functions for replication and packaging of the amplicon but they themselves cannot be packaged. Such systems are relatively free of contaminating helper virus and thus cause minimal cytopathic effects (Fraefel et al. 1997). Problems arose however both from the difficulty in their creation, and the instability of cosmid clones. To overcome these problems the entire 152kb HSV-1 genome minus the packaging signals, was cloned as a bacterial artificial chromosome (BAC) (see figure 1.9) (Saeki et al. 1998; Stavropoulos and Strathdee 1998). These BACS, which package amplicons effectively, are highly advantageous in their stability, simplicity and increased vector titres. However they still often contain low level contamination with replication competent virus.

Recently attempts have been made aimed at long term expression by mediating amplicon retention within the host cell. This has been attempted either by the incorporation of EBV nuclear localisation signals into the amplicon (Wang and Vos 1996) or by the incorporation of AAV ITRs into the amplicon (Costantini et al. 1999; Johnston et al. 1997).
Chapter 1 Introduction

A. HELPER VIRUS

AMPLICON PLASMID

Gene of Interest

HSV oriS

E.Coli ori

HSV packaging "a" sequence

HELPER VIRUS

(Wild Type or Mutant)

orL / oriS

E.Coli ori

TRANSFECTION

INFECTION

DNA Replication

Cleavage and Packaging

Mixed Population of Defective and Helper Virus

RELEASE

Passage of Defective and Helper Progeny

50%

Approximately Equal Mixture of Defective and Helper Virus

B. COSMID BASED

"a"

orL

E.Coli ori

Permissive Cell Lines

~100% Defective Virus

C. BAC BASED

"a"

HSV oriS

E.Coli ori

Permissive Cell Line

~100% Defective Virus

Figure 1.9. Packaging of defective HSV vectors. A) Helper virus dependent packaging system. The therapeutic gene is cloned into an amplicon plasmid which is then transfected into a permissive cell line along with the helper virus. The helper virus provides the replication requirements for both its own DNA and that of the amplicon plasmid. DNA is then packaged separately into new viral particles, that arising from the amplicon can contain multiple copies of the therapeutic gene. After passage on permissive cells the defective virus is in equal abundance to the defective virus. The two types of virus cannot be separated. B) Cosmid based packaging system. Permissive cells are co-transfected with amplicon and a cosmid set spanning the entire HSV-1 genome, minus the packaging sequences. HSV -1 genomes arising form the cosmid cannot be packaged and thus only defective vectors are produced. C) BAC-based packaging system. Permissive cells are co-transfected with amplicon and a BAC containing the entire 152kb HSV genome, minus the packaging sequences. As with the cosmid system only defective viral particles can be packaged because the cosmid lacks cis-acting elements required for its own packaging.
1.4.4.3. Long-Term Transgene Expression from HSV-1 Vectors.
The first reports of HSV-1 vectors carrying transgenes were reported in the late 1980s and early 1990s (Ho and Mocarski 1988; Palella et al. 1989; Chiocca et al. 1990b; Fink et al. 1992). In these experiments the transgene was placed under the control of various different viral promoters, none of which were associated with LAT transcription. For example the ICP4 or ICP8 promoter driving β-gal in the tk locus (Ho and Mocarski 1988), the gC promoter driving β-gal in the US3 gene (Fink et al. 1992), or β-gal in place of the ICPO, ICP4 or tk genes driven by their respective promoters (Chiocca et al. 1990b). In each case transgene expression was transient and no expression was observed in the long term, probably due to the silencing of non-LAT promoters during latency by an undefined mechanism. In contrast, a virus with an insertion of an MMLVLTR/β-galactosidase reporter cassette into ICP4, was able to drive long-term lacZ expression during latent infection in a few cells in the sensory ganglia of a mouse for at least 24 weeks (Dobson et al. 1990). The same virus showed expression in the rat CNS for up to six months, also in a few cells (Bloom et al. 1995). Subsequently the same cassette inserted into the gC locus was shown not to direct detectable levels of β-galactosidase activity during latency, although it was active during acute stages infection (Lokensgard et al. 1994). It was hypothesised that the position of the transgene cassette in ICP4 meant that it was only approximately 500bp downstream of the primary LAT polyadenylation signal and that continued expression might be maintained by transcriptional read-through from the LAT promoters.

The expression of LATs during latency provides the potential for the development of vectors for long term transgene expression, either by direct insertion of transgenes into the LAT region or by the use of LAT promoters at ectopic sites in the HSV-1 genome. In 1989 it was shown that a recombinant virus, with an insertion of a β-galactosidase gene 136bp downstream of the transcription start site of the 5'end of the 2kb LAT, gave lacZ activity up to 8 weeks in mouse sensory ganglia (Ho and Mocarski 1989). The X-Gal staining pattern was low level and punctate suggesting a localisation of β-galactosidase to vesicles or other cytoplasmic structures. The reason for this unusual staining
pattern has not yet been explained and this is a different staining pattern to that found with viruses described later. However the same virus when grown in Vero cells showed no β-galactosidase expression. This provided the first real indication that the LAT promoters could function to drive long term gene expression *in vivo* from a HSV-1 vector. However, the exact elements of the LAT region involved in driving long-term transgene were still not defined and further work to increase efficiency was required. Margolis *et al.* (Margolis *et al.* 1993a) created HSV-1 vectors expressing either β-galactosidase or NGF downstream of the LAP1 TATA box and in place of what has now know as LAP2. They found that transgene expression in both cases was observed 4 days post inoculation but, by the establishment of latency at 21 days, no β-galactosidase or NGF transcripts could be detected. This indicated that the LAP1 promoter alone was not capable of driving long-term transgene expression. Similar results were obtained when a series of HSV-1 deletion viruses were made containing LAP1//lacZ expression cassettes placed in the gC locus (Dobson *et al.* 1995). The viruses contained sequential deletions of the 5' end of LAP1 and in all cases they failed to show sustained transgene expression in sensory neurons in the long-term. Work by Lokensgard *et al.* (Lokensgard *et al.* 1994) showed that fusion of LAP1 upstream to the MMLVLTR promoter and insertion at the gC locus resulted in increased β-galactosidase activity during lytic replication (compared to a vector without LAP1) and also expression of β-galactosidase during latency in DRGs. They tested a number of different promoter/reporter constructs in the gC locus and the recombinant virus containing LAP1/MMLV LTR//lacZ was the only vector to give long-term activity in mouse sensory neurons, as detected by lacZ transcripts at 42 days. Since the LAP1 fragment used did not contain a TATA box, this would indicate that LAP1 conferred long-term expression capabilities on the MMLVLTR, which could not sustain latent gene expression when alone, but this did not occur with other promoters. The MMLVLTR promoter has also been used in a number of other experiments. It was found that by placing MMLVLTR upstream to the LAP1 promoter and in the opposite direction, it could drive long-term β-galactosidase expression up to 18 months, but in very
few cells. This phenomenon was again found to be specific to the MMLVLTR promoter (Carpenter and Stevens 1996).

Following this work a second promoter region (LAP2) was identified in the LAT region (Goins et al. 1994). This had weak activity when used to drive β-galactosidase expression in gC and this weak activity was reported to continue in the long-term. Further work by Lokensgard et al showed that LAP2 could allow LAP1 to remain active in the long-term (Lokensgard et al. 1997). After showing that LAP1 alone was not active during latency (Lokensgard et al. 1994) they produced a set of viruses that contained β-gal reporter cassettes in the gC locus. These cassettes contained LAP1 plus a 1.1 kb downstream long term element, LTE (this essentially contained ~600bp of LAP2 as used by Goins et al). Using RNAse protection assays for the detection of β-galactosidase transcripts in mouse trigeminal ganglia, they showed that the downstream element (LTE) functioned best in conferring long term activity when placed in its natural position, 3' to LAP1. However partial function was obtained when it was inserted upstream of the LAP1 promoter in the reverse orientation, as observed by X-gal staining for at least 28 days.

Subsequent to these findings and by performing similar experiments it was found that LTE could function bi-directionally and could enhance the effect of the LAP1 and tk promoters (Berthomme et al. 2000). Experiments were conducted by placing the LTE in an intron and positioning it in a series of expression cassettes either in the forward and reverse orientation downstream of LAP1 or tk promoters, and upstream of lacZ. Here the β-galactosidase activity was measured by X-Gal staining rather than RNase protection assays. Using this approach it was found that the LTE region (both in the forward or reverse orientations) substantially increased LAT and tk activity, both in the context of plasmid DNA in transient transfection assays and in the context of viral DNA (in the gC locus). They concluded that the LTE has a bi-directional enhancer function on promoters other than LAP1. Using the LAP1/LTE/lacZ viruses in vivo (with LTE in either forward or reverse orientations), it was shown that lacZ expression could be detected at 60 days and 6 months pi, albeit at lower levels than 4 days pi. indicating that LTE can confer long-term transgene
expression in a bi-directional manner. We have reported similar work, some of
which is shown in this thesis, although slightly different conclusions have been
drawn (Palmer et al. 2000). Here it is suggested that a region including LTE can
confer long-term activity on promoters in a relatively promoter non-specific
fashion.

Various other experiments have been performed to achieve long term
transgene expression employing different strategies. It was reported that
viruses containing an internal ribosome entry site (IRES) downstream to the
LAT promoters and before a \textit{lacZ} gene, facilitated the transcription of a reporter
gene without disrupting the LAT promoter region (Lachmann and Efstathiou
1997a). This vector gave long-term transgene expression in both the PNS and
CNS. In the PNS at later times post infection and consistent with the
establishment of latency, an increase in the number of neurons expressing β-
galactosidase and the intensity of staining was observed. However, when tested
in the CNS only a very small number of cells retained expression. The same
group utilised the IRES/β-gal insert to demonstrate that viral vectors impaired
for IE gene synthesis are capable of both establishing latency in sensory
neurons and efficiently expressing a transgene for long periods (Marshall et al.
2000). This strategy combines the use of the LAT region to drive long-term
transgene expression whilst at the same time reducing virus cytotoxicity by the
reduction of IE gene expression. The actual deletion of IE genes has also been
attempted as a means to minimise toxicity and to facilitate long-term transgene
expression (Krisky et al. 1998). However as yet, other than the work from our
laboratory (Palmer et al. 2000; Lilley et al. 2001), none has met with the same
promise as the work of Marshall et al. Using a totally different strategy
Makarova et al (Makarova et al. 1996) employed the use of matrix attachment
regions (MARs) in an attempt to insulate viral promoters from repression during
latency. However their strategy was unsuccessful in the rat brain.

Thus in conclusion, LAP1 is capable of driving short-term reporter gene
expression from its native location or from an ectopic site in the genome. LAP2
is capable alone or in conjunction with LAP1 of directing stable transgene
expression in the long-term. It appears that LAP2/LTE has the ability to confer
an enhancer/supporter function on neighbouring LAP1 and that MMLVLTR may have similar properties.
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D.

Di) (Lokensgard et al. 1994)

\[
\begin{align*}
\text{Ddel} & \quad \Delta gC \\
\text{MMLVLTR} & \quad \beta\text{-gal}
\end{align*}
\]

Dii) (Lokensgard et al. 1994)

\[
\begin{align*}
\text{LAP1} & \quad \text{plasmid} & \quad \text{MMLVLTR} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{Ddel} & \quad \text{Ddel}
\end{align*}
\]

Diii) (Goins et al. 1994)

\[
\begin{align*}
\text{LAP2} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{PstI} & \quad \text{BamHI}
\end{align*}
\]

Div) (Lokensgard et al. 1997)

\[
\begin{align*}
\text{LTE} & \quad \text{LAP1} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{PstI} & \quad \text{BstEII} & \quad \text{SmaI} & \quad \text{SacI}
\end{align*}
\]

Dv) (Berthomme et al. 2000)

\[
\begin{align*}
\text{LAP1} & \quad \text{LTE} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{SmaI} & \quad \text{SacI} & \quad \text{PstI} & \quad \text{KpnI}
\end{align*}
\]

\[
\begin{align*}
\text{LAP1} & \quad \text{SD} & \quad \text{LTE} & \quad \text{SA} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{SmaI} & \quad \text{SacI} & \quad \text{PstI} & \quad \text{HpaI}
\end{align*}
\]

\[
\begin{align*}
\text{LAP1} & \quad \text{SD} & \quad \text{LTE} & \quad \text{SA} & \quad \beta\text{-gal} & \quad \text{pA} \\
\text{SmaI} & \quad \text{SacI} & \quad \text{PstI} & \quad \text{HpaI}
\end{align*}
\]
Chapter 1

Figure 1.10. Schematic diagram to show long-term expression cassettes used in HSV vectors. A. Diagram of the LAT region showing two latency associated promoters, LAP1 and LAP2. Significant restriction sites have been shown. Bi-iv. Long term cassettes that have been inserted in the LAT region. C. Long term cassette that has been inserted into ICP4. Di-v). Long term cassettes that have been inserted into the glycoprotein C locus (gC). MMLVLTR Moloney murine leukaemia virus LTR. β-gal, β-galactosidase. pA polyadenylation signal. IRES, internal ribosome entry site. LTE, long term element. References for each of the cassettes has been shown.
1.5. THE PERIPHERAL NERVOUS SYSTEM - A BRIEF OVERVIEW.

The peripheral nervous system (PNS) is a means of communication between the brain and the external environment and comprises of the cranial and spinal nerves (see later). Each of these nerves consists of parallel bundles of nerve fibres, the axons of which are afferent or efferent (in relation to the CNS) and are either myelinated or non-myelinated.

A commonly used classification system used to identify peripheral nerve fibres is based on the diameter of the fibre, including the myelin sheath where present (see table 1.3.). Peripheral nerve fibres can also be classified according to their speed of conduction of electrical impulses. The smallest fibres, C fibres, have the slowest rate of conduction. The myelinated fibres, A and B, have conduction rates that increase with diameter. Adapted from BARR'S *The Human Nervous System- An Anatomical Viewpoint*, (1998). Kiernan JA and *The Peripheral Nervous System: Structure, Function and Clinical Correlations*, (1987). Mathers LH.

<table>
<thead>
<tr>
<th>Group</th>
<th>External Diameter ($\mu$m)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated Fibres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A $\alpha$</td>
<td>12-20</td>
<td>Motor, skeletal muscles</td>
</tr>
<tr>
<td>A $\beta$</td>
<td>10-15</td>
<td>Sensory, touch, pressure, vibration</td>
</tr>
<tr>
<td>A $\gamma$</td>
<td>3-8</td>
<td>Muscle spindle</td>
</tr>
<tr>
<td>A $\delta$</td>
<td>3-8</td>
<td>Pain (Sharp localised), temperature, touch</td>
</tr>
<tr>
<td>B</td>
<td>1-3</td>
<td>Preganglionic autonomic</td>
</tr>
<tr>
<td>Unmyelinated Fibres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.2-1.5</td>
<td>Pain (diffuse, deep), temperature, postganglionic autonomic</td>
</tr>
</tbody>
</table>

1.5.1. Functional Classes of Peripheral Nerves.

All peripheral nerves are either motor or sensory. The motor nerves are efferent and carry electrical impulses away from the CNS. Sensory nerves are afferent and convey sensory information towards the CNS. There are two categories of efferent neurons; somatic efferents that innervate striated muscle only and visceral efferents that innervate smooth muscle and are part of the autonomic nervous system (sympathetic and parasympathetic). There are two categories of sensory afferents, somatic and visceral. The somatic sensory axons innervate skin, subcutaneous tissues, muscle, connective tissue and joints. Visceral sensory afferents innervate internal organs (viscera), blood vessels and certain glands.

1.5.2. Spinal Nerves.

There are 31 pairs of spinal nerves; 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal. (see figure 1.10.). Each nerve is formed by the union of the ventral and dorsal roots just distal to the dorsal root ganglion. The ventral and dorsal roots connect respectively to the dorsal and ventral horns of the spinal cord. The ventral root consists of bundles of efferent nerve fibres, the dorsal root consists of afferent fibres. The spinal nerve is the only element that contains all of the motor and sensory axons leaving and entering a given spinal segment.

1.5.3. Cranial Nerves.

There are 12 pairs of cranial nerves. All the nerves are distributed through the head and neck except for the vagus nerve, which supplies structures in the abdomen and thorax.
Figure 1.11. Schematic diagram representing the peripheral nervous system at the L4 and L5 level. Motor nerves are seen in red, sensory nerves are seen in blue. Sensory nerves convey information towards the CNS and their cell bodies are located in the dorsal root ganglion. Motor nerves convey information away from the CNS and their cell bodies are located in the grey matter of the spine. Adapted from BARR’S The Human Nervous System- An Anatomical Viewpoint, (1998).
1.5.4. Dorsal Root Ganglion.
The dorsal root ganglia are ovoid structures consisting of pseudounipolar nerve
cell bodies from both somatic and visceral sensory nerves (see figure 1.10).
Each cell body puts out a single process that divides into a central and
peripheral process. The central process travels via the dorsal root to the spinal
cord where it terminates in the grey matter of the dorsal horn. The peripheral
process terminates in a series of dendrites in a peripheral sensory ending.
There are no synapses in the dorsal root ganglia.

1.5.5. Motor Neuron Cell Bodies.
The ventral root of the spinal cord contains the cell bodies of somatic motor
neurons (see figure 1.10.). The axons of these neurons pass into the ventral
root then into the spinal nerve and terminate in motor end plates on skeletal
muscle fibres.

1.5.6. Gene Therapy in the PNS.
In the peripheral nervous system there are a number of applications and
potential disease targets suitable for viral vectors. For example, the treatment of
ALS (amyotrophic lateral sclerosis) (Sendtner 1997), the study of neuronal
development, neuroprotection, nerve regeneration and the investigation and/or
treatment of various pain states (reviewed in Hermens and Verhaagen 1998).

Many neurodegenerative disorders are characterised by the death of specific
neuronal populations. Mostly these disorders arise in the CNS, such as
Parkinson's disease and Alzheimer's disease however those of the PNS include
ALS and peripheral neuropathies. As yet there seems to be no effective
therapies for such complaints and consequently the use of neurotrophic factors
is being pursued (Walsh 1995). Many neurotrophins stimulate neurite
outgrowth, suggesting that they may promote regeneration of injured neurons
(Davies 1994). For the treatment of ALS, several neurotrophic factors are
known to positively influence motor neurons both in vivo and in vitro, such as
ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3) and glial derived
neurotrophic factor (GDNF) (Davies 1994). GDNF is the most potent known
survival factor for motor neurons in vitro (Lapchak et al. 1996). Peripheral
neuropathies include chemotherapy-induced neuropathy, diabetic neuropathy and post polio syndrome. It is known that peripheral and sympathetic neurons depleted in peripheral neuropathy respond to nerve growth factor (NGF) treatment (Walsh 1995).

Herpes simplex virus is potentially an excellent tool for gene delivery to the PNS for a number of reasons (see section 1.4.3.). Since many disease treatments are likely to involve combination therapy with different molecules, one of the most pertinent features of HSV-1 as a gene therapy vector is its large insertional capacity for foreign genetic material. In addition, the HSV-1 natural lifecycle involves infection of peripheral nerves and the virus has evolved the ability for retrograde and anterograde transport along the axon. The inability to move retrogradely is a problem observed in other viral vector systems, where the only means to achieve substantial gene delivery to the PNS is via direct injection into the preferred target such as the DRG (Glatzel et al. 2000). HSV has also evolved the ability to enter lifelong latency in spinal ganglia. For these reasons we and others have chosen to develop HSV-1 as a vector for gene delivery to the peripheral nervous system.
1.6. INDUCIBLE GENE EXPRESSION.

The technology required to precisely control gene expression in mammalian cells has been extensively studied over the last decade. Successful control of the expression of delivered genes will provide a means to study complex physiological processes and provide information on gene function and regulation throughout development. Gene therapy vectors that incorporate such technology are likely to have a huge impact on the efficacy of future gene therapy procedures. Previously, the use of traditional knockout or transgenic technology as a method to study gene function meant that if the complete loss of a gene product results in embryonic lethality, then gene function at later stages of development could not be studied. Temporal and spatial control of gene expression would overcome this problem and additionally such regulatable systems will allow the study of cytotoxic gene function. In terms of gene therapy protocols, many diseases may require the temporal control of gene expression, as over-production of the delivered protein will often be detrimental. Examples include diabetes, thalassemia and neuropathy. Thalassemia, which is caused by mutations in the genes that code for haemoglobin, results in a deficiency in α and β globin relative to one another (Higgs 1993; Thein 1993). Unregulated over-expression of say β-globin, will lead to the conversion of β-thalassemia (relative lack of β-globin) into α thalassemia (lack of α-globin), and thus conversely over expression of α-globin could convert α-thalassemia into β-thalassemia. Type-1 hereditary sensory-motor neuropathy and tomaculous neuropathy share an interesting disease profile since either the excess or reduction in the levels of peripheral myelin protein 22 respectively leads to abnormal peripheral myelination (Thomas and Harding 1993). Thus in all these cases there is a clear need for regulated gene expression as an inappropriate excess or lack of gene product cannot be tolerated.

Initial inducible gene expression systems developed for use in eukaryotes involved the use of endogenous promoters and enhancers, responding to signals such as heat shock, heavy metal ions or hormones. However such systems in general were found to be unsuitable due to the pleiotrophic effects
common to them all. As a result not only the required gene, but also other genes responded to induction, making interpretation of results somewhat problematical (Gingrich and Roder 1998).

An inducible system would ideally possess a number of characteristics: low basal expression in the uninduced state; rapid induction kinetics, that are dose responsive and can be efficiently reversed; components should not respond to or interfere with endogenous factors; the inducer should be safe and easy to administer. To avoid the pleiotrophic effects of induction the utilisation of systems that are not endogenous to mammalian cells became an obvious solution. This has been achieved through the use of prokaryotic regulatory elements. A number of promising prokaryotic ligand-inducible systems have now been developed, but for the purposes of this thesis I will describe the three most common, and which were used later, in detail.

1.6.1. The Tetracycline Inducible System.
The tetracycline inducible system is the most extensively characterised and used regulatory system today. The original system was developed by Gossen and Bujard and is based on the tetracycline resistance operon, encoded by the Tn10 transposon of E Coli. (Gossen et al. 1995; Gossen and Bujard 1992). The system comprises of a fusion between the tet repressor (TetR) protein and the C-terminal transactivation domain of the HSV protein VP16. This produces a chimeric protein known as tTA, the tetracycline controlled transcriptional transactivator. The second component of the system is the Tet response element (TRE) which contains the gene of interest cloned downstream to tet operators fused to a human CMV minimal promoter. In the original 'Tet-Off' system, (Gossen and Bujard 1992) binding of Tet to tTA causes a conformational change in the protein, which prevents it binding to the operator sequences in the TRE and thus this leads to inactivation of gene expression. In the 'Tet-On' system a change of 4 amino acids reverses the affects of tTA, now termed rtTA, such that tetracycline binding allows interaction with the TRE and thus reporter gene expression is switched on (Gossen et al. 1995) (see figure 1.12). The development of rtTA was prompted by the observation that the continuous presence of ligand in order to repress transgene expression, as with
tTA, is sub-optimal both for transgenics and gene therapy. Initial in vitro experiments using stable Hela cell lines suggested that the ‘Tet-Off’ and ‘Tet-On’ systems produced transgene regulation up to five and three orders of magnitude respectively. However since these initial findings using Hela cells, it has been reported that the efficacy of at least the ‘Tet-Off’ system is highly cell type specific (Ackland-Berglund and Leib 1995; Paulus et al. 1996). The systems have subsequently been successfully used in transgenic animals, both in terms of efficacy and tissue specific gene expression (Kistner et al. 1996; Mayford et al. 1996; Furth et al. 1994).

A number of viral vectors incorporating the elements of either the ‘Tet-On’ or ‘Tet-Off’ systems have been documented, including retroviral, lentiviral, adenoviral, AAV and HSV amplicon systems. The most successful, in terms of levels of gene induction both in vivo and in vitro, has been with an Ad vector (Harding et al. 1997; Harding et al. 1998). This work used E1 deleted Adenovirus vectors and tested both Tet-Off and Tet-On systems in vitro on primary hippocampal cultures and in vivo in the rat hippocampus. In both the systems two viruses were used, one containing the transactivator and the other containing the tet response element. In vitro they found that the AdTet-Off showed a 331-fold repression and the AdTet-On a 119-fold induction of EGFP expression, as determined by fluorimetric analysis of cell lysates. In vivo the group showed that both the AdTet-On and AdTet-Off vectors could persist in the rat hippocampus for at least three months and that within this period gene expression could be switched on/off and then on again. It is interesting to note that in vivo the viruses had to be injected at a ratio of 1:20 (transactivator virus/Ad-TRE) for efficient gene regulation to occur. Subsequent to these findings, the same group has used the ‘Tet-Off’ system to mediate cell specific gene expression in neuronal and glial cell populations, both in vitro and in vivo (Ralph et al. 2000). Other groups, using a tet regulable retroviral vector to achieve cell specific expression have not met with similar success (Paulus et al. 1996). There have been a few reports of ‘Tet-Off’ inducible, HSV amplicon mediated, gene expression (Fotaki et al. 1997; Ho et al. 1996).
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Figure 1.12. Schematic representation of the tetracycline inducible expression systems. A. Tet-Off. B. Tet-On (reverse Tet). The transactivator consists of the VP16 transactivation domain fused with either the tetracycline repressor, tetR, or the reverse rtetR. In this example transactivator expression is driven by the human CMV promoter (pCMV). A. In the Tet-Off system the presence of tetracycline or doxycycline renders the tTA unable to bind to the tet operator sites and the reporter gene is silenced. B. In the Tet-On system the situation is reversed. Doxycycline binds to the Tet repressor and allows the rtTA to bind to the seven operator sites, allowing reporter gene expression from the minimal CMV promoter. Doxycycline activates rtTA to a greater extent than tetracycline.
This work used either an ICP4 or an ICP27 deleted HSV helper virus. The maximum repression observed in either of these cases was 60-fold following injection into the adult rat hippocampus. The studies showed that these amplicon vectors were responsive to tet in a dose dependent manner (Ho et al. 1996), however the major drawback in each case was the rapid decline of transgene expression between 2 days and a week. This impeded further characterisation of the systems. Since it was reported in at least one of the accounts that the amplicon vector/helper vector ratio was 1:30, and could not be improved (Ho et al. 1996), it could be speculated that helper virus toxicity could be a major concern and relate to the drop in gene expression (Johnson et al. 1992).

Utilising different approaches several other groups have successfully using tet regulatable viral vectors. Similarly to the work of Harding et al (Harding et al. 1998) the two vector approach was used in AAV vectors (Rendahl et al. 1998). Recombinant AAV vectors containing either transactivator or the tet response element were co-injected into mouse skeletal muscle and regulated expression was observed over an 18-week period. In contrast to this two-vector approach, a self-inactivating retroviral vector containing a single autoregulatory cassette of the Tet-Off system also proved successful in vitro (Hofmann et al. 1996). In this novel system the vector encoded a bicistronic mRNA allowing expression of both β-gal and tTA from tetO-pCMV, with tTA translation being initiated at an IRES. Other groups have also utilised a single retroviral vector approach (Paulus et al. 1996). Bohl et al (Bohl et al. 1997) achieved 70-fold dox regulated control of erythropoietin secretion in mice after an ex vivo approach transducing myoblasts with retroviruses containing rtTA and inducible erythropoietin. However in this system high basal expression proved problematic.
1.6.2. A Novel Tet Repressor System.

Recently a novel tetracycline regulatable expression system has been documented (Yao et al. 1998). In this system the tetracycline repressor (tetR) alone, rather than tetR-mammalian cell transcription factor fusion derivatives, can function as a potent modulator of gene expression. See figure 1.13.

Figure 1.13. Schematic representation of the tetR-mediated transcriptional repression switch. A. In the absence of tetracycline the tetR dimer binds to the tet operator bearing hCMV major immediate early promoter. This interaction prevents the assembly of the transcription initiation complex, leading to no gene expression. B. When tetracycline is present the ability of tetR to interact with tetO is abolished. Thus, the tetR-mediated repression is released and gene expression can begin.
Yao and colleagues created a response element consisting of two tet operator sequences inserted 10bp downstream of the last nucleotide of the TATA element of the hCMV promoter. Downstream to this they inserted the human epidermal growth factor gene (hEGF). They determined that reporter gene expression was not compromised by the insertion of the operator sequences and that tetR alone could function as a potent repressor of gene expression. This was presumably by blocking either the transcription initiation complex interacting with the CMV promoter or the TBP interacting with the TATA element. In the presence of tetracycline, the tetR is prevented from binding to the operator sequences and gene expression proceeds. In transient transfection assays a 1500-fold repression was seen on Vero cells and similarly to the Tet-Off system (Ackland-Berglund and Leib 1995) this repression was shown to be cell type specific. It was also determined that the molar ratio of tet repressor to response element (pcmvtetO) was critical, and that at a ratio of 6:1 (repressor/response element) improved repression could be observed. One of the advantages of this system is that in the resting state the tetR binds to the operator sequences and thus in theory no basal leakiness should be observed or at least should be minimal. However in the initial reports repression was somewhat leaky (Yao et al. 1998).

Thus far there have been no reports of this tet repressor system used in the context of a viral vector. However the system has been used as a novel tool to produce a tetR-mediated HSV-1 viral replication switch (Yao and Eriksson 1999a). Here the repression system was used to control the expression of a mutant form of the essential HSV-1 replication gene UL9, a gene involved in binding to HSV-1 origins of replication. The mutant protein prevents viral replication by suppression of viral protein synthesis. The system was shown to be functional in vitro and it was suggested that the strategy could be used in terms of a HSV-1 viral vector, capable of inhibiting its own replication as well as the replication of wild-type virus (Yao and Eriksson 1999b).
1.6.3. The Ecdysone Regulated System.

Ecdysone is a steroid hormone that functions to stimulate metamorphosis in insects such as *Drosophila melanogaster*. The ecdysone receptor was identified in the early 1990s (Yao *et al.* 1993) and using this information an ecdysone inducible gene expression system was produced (No *et al.* 1996). In *Drosophila* the ecdysone receptor (EcR) mediates its function by forming a hetero-dimer with the product of the ultraspiracle gene (USP) (Yao *et al.* 1992). The original ecdysone inducible system involved co-expressing the genes for EcR and USP, with an ecdysone-responsive reporter construct. However, induction levels with this system were only 3 fold (Yao *et al.* 1992; Yao *et al.* 1993). The system was then modified, through a number of steps, to give a complex that produces inducibility in stable cell lines approaching three orders of magnitude (No *et al.* 1996). This system uses the VgEcR protein, an N-terminal truncation of EcR, which is fused to the C-terminal transactivation domain of HSV VP16. This chimeric ecdysone receptor is incapable of DNA binding or transcriptional activation and can only do so when it heterodimerises with its counterpart the retinoid-X-receptor (RXR). RXR is a mammalian homologue to USP. The VgEcR/RXR dimer forms a transactivator complex when bound to the appropriate DNA response element. The response element constitutes four copies of a hybrid ecdysone/gluccocorticoid response element (4x E/GRE), upstream of a minimal promoter which drives the transgene of interest. Ecdysone binding to the transactivator mediates an interaction with the response elements and thus transactivation and expression of the gene of interest (see figure 1.14.).

Once developed, this system was shown to be effective in the context of transgenic mice, directing the tissue specific inducible expression of β-galactosidase (No *et al.* 1996). Although the authors did not quantify the levels of induction, basal expression of β-galactosidase appeared low. Indeed, in a direct comparison between the ecdysone inducible and tetracycline inducible systems, using transient transfection assays on CV-1 cells, 20 fold (Tet-Off) and 500 fold (Tet-On) improved basal activities were observed compared with the ecdysone system. However this observation may be a result of the cell type used, since the efficacy of the 'Tet-Off' system is know to be cell type
dependent. Subsequently an ecdysone system was used in order to achieve mammary gland-directed, ligand inducible expression in transgenic mice (Albanese et al. 2000). Mammary gland specific expression was directed by using the MMTVLTR promoter to drive VgEcR expression. The group demonstrated both sustained and tissue specific regulation of reporter gene expression, with the intent to utilise the system to facilitate mammary gland tumorigenesis studies.

The only reports of ecdysone inducible viral vector mediated gene expression utilise either retroviral or adenoviral vectors (Suhr et al. 1998; Hoppe et al. 2000). Both utilised an ecdysone receptor from Bombyx mori (BmEcR) which, in contrast to the EcR from Drosophila melanogaster, is capable of full transactivation with no added exogenous RXR. Shur et al used retroviral vectors as tools to create stably transfected cell lines (293 cells, CV1 cells and rat primary fibroblasts) in order to characterise a BmEcR/VP16 hybrid. They found that this hybrid had potent induction capabilities in the absence of superphysiological levels of RXR. Subsequently Hoppe et al used this finding and created a chimeric Drosophila/Bombyx ecdysone receptor (DB-EcR) that preserved the ability to bind to the modified ecdysone promoter E/GRE but did not require exogenous RXR for effective transactivation. When compared directly to VgRXR, DB-EcR (in the absence of exogenous RXR) exhibited lower basal transactivation levels and was capable of effectively mediating inducible gene expression. Additionally in vivo after intramyocardial injection of a mixture of two Ad vectors containing either DB-EcR or separately a reporter cassette, a 40-fold induction of luciferase activity was measured after ligand induction (Hoppe et al. 2000). Again this shows the benefit of a two-vector approach, although here the authors used the Ads at a ratio of 1:1. Further improvements may be observed if the ratio was optimised.

Ecdysone has a number of analogues that are more commonly used, including muristerone A (MurA) and ponasterone A (PonA). An advantage of this system is that these and other ecdysteroid compounds are generally lipophilic in nature and so can readily penetrate all tissues, including the brain. Additionally components of the system are non-endogenous to mammalian cells and thus
are unlikely to effect the physiology of the cells. Unfortunately, one of the severe limitations of the ecdysone system is the availability of the ecdysone analogues. The popularity of the system has lead to the unexpected decline of muristeroneA stocks, the originally used ecdysone analogue. This ligand is obtained from a plant native to certain slopes of the Himalayas, and as such is inaccessible and costly. Investigations into possible steriodal and non-steroidal alternatives to MurA lead to the identification of PonA as having the best induction capabilities (Saez et al. 2000). Unfortunately this is also very expensive and in short supply. This has lead groups to isolate their own PonA (from species of the Podocarpus or Taxus genus of evergreen plants, such as Podocarpus macrophyllus) instead of commercial purchase (Albanese et al. 2000). This situation was found to be far from ideal for the work described in this thesis using the ecdysone inducible system, particularly in vivo.
Figure 1.14. Schematic diagram of two ligand inducible gene expression systems

A. The ecdysone system. B. The progesterone antagonist (RU486) system.

A. The ecdysone transactivator consists of a heterodimer between hRXRa and a chimeric protein, VgEcR, consisting of a modified EcR fused to a VP16 transactivation domain. In the presence of ecdysone or an analogue e.g. ponasterone A, the transactivator complex binds to a hybrid response element consisting of ecdysone/glucocorticoid response element half sites (E/GRE). Reporter gene expression is driven by a minimal promoter.

B. The RU486 transactivator consists of a yeast GAL4 DNA binding domain fused to a truncated human progesterone receptor ligand binding domain (PR-LBD) and a VP16 transactivator domain. In the presence of RU486 the transactivator binds to an inducible promoter consisting of 4xGAL4 DNA binding sites. Reporter gene expression is driven by an adenovirus E1B minimal promoter.
1.6.4. The Progesterone Antagonist System.

The existence of a C-terminal deletion mutant of the human progesterone receptor has previously been described (Vegeto et al. 1992). This receptor is incapable of binding its agonist progesterone, but paradoxically retains its ability to bind progesterone antagonists such as RU 486 (mifepristone). Using this information a regulatory system was designed based on the mutant receptor (Wang et al. 1994). The system comprises of the mutant ligand binding domain of the progesterone receptor (PR-LBD), fused to the binding domain of the yeast transcriptional activator GAL4 and the herpes simplex protein VP16 transactivation domain. This chimeric construct is the transactivator (GL-VP) and in the presence of RU486, or other progesterone antagonists, it functions to transactivate a response element consisting of four copies of the GAL4 binding site. The response element is located upstream of a minimal TATA containing promoter element, derived from the adenovirus E1B gene, and a reporter gene. Activation via the response element causes induction of gene expression. (see figure 1.14.)

The efficiency of this system was demonstrated in vitro using transient transfection assays, showing a 50 fold induction of the CAT reporter gene upon addition of RU486 (Wang et al. 1994). Using an ex vivo approach the authors demonstrated an 8-10 fold induction of TH after intramuscular transplantation of stable cell lines containing both the regulator and reporter construct into rats. This was following oral administration of RU486. Subsequent experiments tested the efficacy of the system in vivo, using bigenic mice expressing GL-VP and an inducible human growth hormone transgene (Wang et al. 1997). Here the transactivator was under the control of the transthyretin promoter (TTR), which directs gene expression to the liver. The double transgenic mice showed up to a 3500-fold induction of hGH in their serum after oral administration with RU486. The system was also shown to be dose responsive to RU486 as measured by serum hGH levels, exhibit low basal expression in the absence of RU486 and inducible after three weeks after pulsed administration of RU486 (Wang et al. 1997). Together these experiments demonstrated the potential of the steroid system.
Recently, the first use of the progesterone antagonist system using a HSV-1 vector was documented (Oligino et al. 1998). As a prerequisite to this, the group verified the ability of a bipartite GAL4/VP16 transactivation construct, to activate reporter gene expression from a minimal GAL4-TATA promoter, in the context of a replication incompetent HSV-1 vector (Oligino et al. 1996). In order to allow regulation an ICP4 deleted recombinant virus containing the full RU486 inducible system, both transactivator and response element was produced. The double recombinant vector contained the transactivator, driven by a CMV promoter, in the tk locus, and the response element in the gC locus. The vector showed a 20-fold induction of β-galactosidase expression in vitro and a 150-fold induction in vivo upon administration of RU486. In vivo experiments were performed in the rat hippocampus. The data showed a ‘proof of principle’ indicating that the RU486 system could be used to induce HSV transduced transgene expression in the CNS. However, as the data was only collected over 48hrs, it is unclear if the induction could be maintained over longer periods or could be switched on/off.

A more successful viral vector approach was achieved using a gutless Ad vector (Burcin et al. 1999a). This used a slightly modified version of the progesterone system, and replaced the VP16 moiety of the transactivation complex with an activation domain of human p65, a member of the NF-κB family. For use in recombinant adenovirus vectors a single regulatory cassette was created containing both transactivator and RU486 response element (Burcin et al. 1999a). Most interestingly the chicken β-globin insulator element was used to separate the two components of the cassette. In vitro the Ad vectors showed dose dependent RU486 induction and the Ad vector containing the insulator elements showed no detectable levels of reporter gene (hGH) in the absence of ligand, a highly attractive feature. In vivo the Ad vectors were shown to achieve RU486 dependent regulation that could be reinduced up to five times over a period of 12 weeks. However in vivo the insulator elements did not show any advantageous effect and only served to reduce the level of hGH expression compared to the Ad virus with no insulator.
In conclusion, the use of RU486 as an inducing compound is attractive inasmuch as it is orally available, lipophilic in nature and has well characterised pharmacokinetic properties. Additionally it readily crosses the blood brain barrier. For successfully integration into gene therapy protocols, RU486 will need to be used at concentrations well below that which it is used in abortion induction protocols (10mg/kg oral dosage). This has been achieved so far in many reported protocols using viral vectors for gene delivery, which \textit{in vivo} used no more than 500\mu g/kg via ip administration (Burcin \textit{et al.} 1999b). However, somewhat alarmingly, in one reported protocol, doses of 25mg/kg RU486 were used (Oligino \textit{et al.} 1998).

1.7. THESIS AIMS.

As mentioned previously HSV is an ideal vector for gene therapy of the PNS. The aim of this thesis is to develop the use of attenuated HSV-1 vectors that are non-pathogenic, non-cytotoxic and able to express transgenes during both lytic and latent infection.

Most disease treatment will require long term gene therapy and thus particular attention will be focused on the latent, thus long term gene expression characteristics of the vectors studied. Recombinant vectors will be constructed that are deleted for various combinations of essential and non-essential genes and their gene delivery profiles compared. Expression cassettes containing the HSV latency associated promoters, LAP1 and LAP2/LATP2, in conjunction with other promoters, will be used to create recombinant vectors. The use of the LAT promoters will be assessed for their ability to support long term transgene expression. Optimised vectors will then be used to construct viruses containing therapeutic genes.

This thesis also aims to create a ligand regulatable HSV-1 vector. Work will concentrate on three ligand inducible systems, the Tet system, the ecdysone system and the progesterone antagonist system. Promising vectors will be tested \textit{in vitro} and \textit{in vivo}. 
CHAPTER 2:

MATERIALS AND METHODS
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2.1. MOLECULAR BIOLOGY.

2.1.1. Laboratory Reagents.

General laboratory chemicals were of analytical grade and purchased from either Merck Ltd, Poole, Dorset, UK; Boehringer Mannheim, Lewes, East Sussex, UK; Difco Laboratories, Detroit Laboratories, Detroit, USA-, Sigma Chemical Company Ltd, Poole, Dorset. Gibco-BRL Life Technologies, Paisley, Renfrewshire, UK or Biometra Ltd, UK Hexanucleotides [pd(N)6} for random primer labelling, and dNTPs were obtained from Pharmacia Biotechnology Ltd, St. Albans, UK. Radiochemicals and Hybond nylon membranes were obtained from Amersham International plc. Little Chalfont, Bucks., UK. Kodak X-OMAT imaging photographic film was purchased from Sigma Chemical Co. Ltd. Kodak Professional 64T colour film was also purchased from Sigma Chemical Company Ltd.

2.1.2. Bacterial Strains and Growth Conditions.

2.1.2.1. Bacterial Strains.

The XL1-Blue (Stratagene Ltd., Cambridge, UK) strain of E.coli was used for all plasmid cloning except where stated.

XL1-Blue  recA1  endA1  gyrA96  thi-1  hsdR17  supE44  relA1  lac
[F'proAB  LacF  ZΔM15, TN 10 (Tet)']

2.1.2.2. Propagation of Bacteria.

XL1-Blue (XL1-B) cells were grown in Luria Bertani (LB) media containing either no antibiotic or 100μg/ml ampicillin overnight in a Gallenkamp orbital shaker at 200rpm. LB media was autoclaved at 120°C for 20mins at 10lb square inch⁻¹. Stocks of ampicillin (Sigma Chemical Company, Poole, Dorset, UK) were made at 100mg/ml (1000x) and stored at -20°C. Colonies of XL1-B were isolated by growing on LB plates containing 2% Bacto®-agar.
Luria Bertani (LB Media) 1% (w/v) Bacto®-tryptone
1% (w/v) NaCl
0.5% (w/v) Bacto®-yeast extract

2.1.2.3. Preparation of Competent XL1-Blue Cells.
Competent XL1-B cells were prepared using a standard calcium chloride technique (Sambrook et al., 1989). A 5ml starter culture (containing no antibiotic) of XL1-B were grown overnight which was then used to inoculate 100mls of LB (containing no antibiotic) and the culture was grown to an OD_{580} of 0.4-0.55 units. The bacteria were then pelleted by centrifugation at 2000rpm for 10mins at 4°C and any excess LB was removed. The cells were resuspended in 10mls of ice-cold 100mM CaCl₂ and incubated on ice for 1hr. The cells were then pelleted as before and resuspended in 4mls of ice cold CaCl₂. The cells were then incubated on ice until required.

2.1.2.4. Transformation of XL1-Blue Cells.
200μl of competent XL1-B cells were transformed by addition of DNA and subsequent incubation on ice for 30mins. The cells were then heat shocked for 90secs at 42°C. The cells were then incubated on ice for a further 2mins. 800μl of LB was then added to the cells and the cells incubated in the orbital shaker for 1hr at 37°C/200rpm. The cells were then pelleted and resuspended in 100μl of LB and plated onto LB agar plates containing 100μg/ml of ampicillin. If detection of β-galactosidase was required, plates containing 50μl of 20mg/ml stock of 4-Cl, 5-bromo, 3-indolyl-β-galactosidase (X-Gal, Insight Biotechnology Ltd.) dissolved in dimethyl formamide, were used.

2.1.3. DNA Extraction Techniques.

2.1.3.1. Small Scale Plasmid DNA Extraction from XL1-B cells.
The “mini-prep” extraction method used is based on an alkaline lysis method described previously (Birnboim and Doly 1979). Single colonies of XL1-B cells were used to inoculate 5mls of LB containing 100μg/ml ampicillin, and were incubated in an orbital shaker overnight, as before (section 2.1.2.2.). The cells
from 1.5ml of culture were then pelleted by centrifugation at 2000rpm for 2mins. The cells were resuspended in 100μl of resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA pH 8, 100μg/ml RNase-A). Cells were lysed by addition of 200μl of lysis buffer (200mM NaOH, 1% (v/v) Triton X-100) and then 150μl of neutralisation buffer (3M NaOAc pH 5.5) was added. The cell lysate was then centrifuged for 5mins at 2000rpm and the pelleted precipitate was removed with a hypodermic needle bent at the tip. 500μl of isopropanol was added to the remaining supernatant and the mixture was then centrifuged for 5mins at 15000 rpm. The supernatant was removed and the DNA pellet was washed with 500μl of 70% ethanol (EtOH). The DNA pellet was air dried and then resuspended in 50μl of double-distilled water (ddH$_2$O). Plasmid DNA was stored at −20°C.

2.1.3.2. Large Scale Plasmid DNA Extraction.

Large scale DNA plasmid extractions were performed to produce high grade DNA for cloning and the isolation of DNA fragments (section 2.1.4.5) and transfections (section 2.2.4.1.). A 10ml overnight culture of transformed XL1-B cells were used to inoculate 400mls of LB containing 100μg/ml ampicillin and incubated in an orbital shaker overnight. 100mls of the culture were then spun down at 3000rpm for 10mins. Plasmid DNA was then extracted using the Qiagen Midi-Prep Kit (Qiagen, Chatsworth, USA) as per manufacturer’s instructions. A typical yield of DNA was 100μg, which was resuspended in 100μl of ddH$_2$O.

2.1.3.3. Small Scale Viral DNA Extraction.

Small-scale viral DNA extractions were performed in order to obtain viral DNA for Southern blot analysis (section 2.1.7.). A 35mm dish of virally infected cells at complete cytopathic effect (CPE) were harvested and pelleted by centrifugation at 1000rpm for 10mins. The cells were resuspended in 200μl of TES buffer (50mM Tris pH 7.8, 1mM EDTA, 30% (v/v) sucrose). 200μl each of solutions A and B (A: 2% (w/v) SDS, B: 100mM β-mercaptoethanol) were then added and the resulting solution incubated on ice for 30mins. 10μl of Proteinase K (stock at 20mg/ml) was then added and the cells were 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 then precipitated by addition of 75μl of 7.5M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol and subsequent centrifugation at 15000rpm for 10mins. The viral DNA pellet was then washed with 500μl of 70% EtOH and the DNA pelleted was air-dried for 5mins. Viral DNA was then resuspended in 50μl of ddH₂O.

2.1.3.4. Large Scale Viral DNA Extraction.

Viral DNA extracted by this method was used in transfections to produce recombinant viral vectors (sections 2.2.4. and 2.2.5.). Virally infected cells at complete CPE were harvested from 4-8 x 175cm² tissue culture flasks. Replication competent and incompetent virus required 4 or 8 flasks respectively. Cells and virus were pelleted by centrifugation at 12000rpm for 2hrs in a Beckman JA14 or equivalent. The supernatant was then discarded and the pellet was resuspended in 10mls of Proteinase K buffer (0.01M Tris pH 8.0, 5mM EDTA, 0.5% SDS) and Proteinase K was added to a final concentration of 50μg/ml. The resuspended pellet was then incubated in an orbital shaker (200rpm, 37°C) overnight or until the lysate became clear. 10mls of ddH₂O were then added to the lysate. Extractions were performed by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by gentle mixing, by inversion, for 10mins. The mixture was then divided equally into Beckman polyallomer tubes and ultracentrifugation performed at 15000rpm for 30mins in an SW41 rotor or equivalent. Repeated extractions steps were performed on the aqueous layer, with ultracentrifugation for 10mins only, until no white protein interface appeared after centrifugation. The aqueous layer was then transferred into 50ml tubes and a final extraction was performed using an equal volume of chloroform/isoamyl alcohol (24:1). The tubes were mixed by gentle inversion for 5mins and spun on a benchtop centrifuge at 2000rpm for 5mins. The aqueous layer was removed and the viral DNA was then precipitated with 2 volumes of room temperature 100% EtOH, by running the ethanol gently down the side of the tube, layering on the aqueous solution and then gently mixing. The precipitated viral DNA was then pelleted by centrifugation at 3000rpm for 10mins. 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 an appropriate volume of ddH₂O and the integrity of the DNA was determined by running 5µl on a 1% agarose gel. Replication competent vector DNA was resuspended in 3mls of water and replication incompetent vector DNA between 100-500µl.

2.1.4. Cloning Techniques.

2.1.4.1. Analytical Restriction Digests.
Restriction digests were performed on plasmid DNA for analytical clonal analysis. Digests were performed in a total volume of 20µl, containing either 5µl of mini-prep DNA (section 2.1.3.1.) or 1µl of midi-prep DNA (2.1.3.2.). 10 units of each enzyme were added and the appropriate buffer as specified by the manufacturer. The volume was made up to 20µl using ddH₂O. Digests were incubated for 1-2hrs at the appropriate temperature. The digests were then run on a 1% agarose gel and bands visualised on a UV transilluminator.

2.1.4.2. Isolation of DNA Fragments.
Restriction digests required for isolation of DNA fragments were carried out in a total volume of 20µl containing ~5µg of midi-prep DNA, 10 units of each enzyme and the appropriate buffer as specified by the manufacturer. The volume was made up to 20µl using ddH₂O. Digests were incubated overnight and then run on a 1% agarose gel. DNA bands were visualised on a UV transilluminator and the required bands were carefully excised using a scalpel. DNA was then extracted from the agarose using the GeneClean II Gel Extraction Kit (Bio 101, Vista, CA, USA) as per manufacturer's instructions. The DNA was then eluted in a final volume of 20µl.

2.1.4.3. Blunt-End Reactions.
When there were no compatible restrictions sites for cloning, sticky-ends were blunted by filling in their 3' overhangs using T4 DNA polymerase (Promega, Southampton, UK.). After restriction digest, 1µl of a 25mM stock of dNTPs (dATP, dCTP, dGTP, dTTP) and 15 units of T4 DNA polymerase was added.
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The reaction was incubated for 30 mins at 37°C. If subsequent restriction digests were to be performed, the reaction was heat inactivated at 80°C for 30 mins and then cooled on ice for 20 mins prior to addition of further restriction enzymes. After blunt-ending, the reaction was run on a 1% agarose gel.

2.1.4.4. Phenol Extraction of Digested DNA.
When DNA restriction digestions needed to be purified, a phenol extraction was performed by the standard technique (Sambrook J 1989). This was necessary if a digestion required two enzymes and a compatible buffer could not be found, or when linearised DNA was used in transfections. Digestions were made up to a total volume of 400 μl with ddH₂O. An equal volume of phenol/chloroform/IAA (24:24:1) was added and the mixture vortexed thoroughly before centrifugation at 12000 rpm for 2 mins. The aqueous layer was removed and an equal volume of chloroform/IAA (24:1) was added (400 μl). The mixture was vortexed thoroughly and pulse spun. The aqueous layer was removed and to this was added 40 μl (1/10th total volume) of 3M NaOAc (pH 5.5) and 880 μl (2 volumes) of ice cold 100% EtOH. The mixture was vortexed and then left at −70°C for 10 mins, before centrifugation for 15 mins at 13000 rpm to pellet the DNA. The supernatant was then removed and the DNA washed in 70% EtOH before being left to dry. The extracted DNA was then resuspended in 10 μl of ddH₂O.

2.1.4.5. DNA Ligations.
Ligations were performed in thin walled 0.5 ml reaction tubes in a total volume of 20 μl. Reactions contained 1-2 μl of gel-purified vector, 7-10 μl of gel-purified insert, 1x ligase buffer, 1-3 units of T4 DNA ligase (Promega, Southampton, UK) in ddH₂O. Reactions were placed either a RT for ~2 hrs or in the case of blunt end ligations in a thermocycler (Eppendorf Thermocycler). The thermocycler reactions were incubated at 16°C for 1 min followed by 37°C for 1 min, for 30 cycles. Then a final incubation at 22°C for 30 mins. In each case the ligation reaction was transformed into 200 μl of competent XL1-B cells as described earlier (section 2.1.2.4.).
2.1.5. Colony Characterisation Using a Radiolabelled Probe.

In the absence of X-Gal mediated selection, bacterial colonies could be screened for the incorporation of an inserted DNA fragment using a radiolabelled probe. This method was of particular use following blunt end ligations where insertional efficiency was usually very low.

2.1.5.1. Colony Transfer.

Bacterial colonies were directly transferred onto Hybond-N+ nitrocellulose membranes by placing membranes onto the LB-agar plates for 2mins. Holes were punched in the filter in an assymetric pattern using a sterile needle. The position of the holes was marked on the petri dish with a waterproof pen for the purpose of orientation at a later stage. The bacterial cells were lysed and the DNA denatured by immersing the nitrocellulose membranes in denaturing solution (1.5M NaCl, 0.5M NaOH) for 2mins. The filters were then immersed in neutralisation solution (2M NaCl, 1M Tris pH 5.5) for 2mins and washed in 2x SSC/0.1% SDS for 2mins. The filters were washed twice in 2x SSC for 2mins. Placing membranes colony side up they were cross-linked twice (UV Stratalinker 2400).

2.1.5.2. Hybridisation.

The nylon membranes were pre-hybridised for between 2-5 hours at 65°C with 30mls of pre-hybridisation solution (6x SSC, 5 x Denhardt’s reagent, 0.5% w/v SDS in ddH2O 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 ddH2O]). The volume of the pre-hybridisation solution was reduced to 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 10mins 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. If necessary the blot was washed further in 0.5x SSC/0.1% SDS and then 0.1x SSC/0.1% SDS.
2.1.5.3. Radiolabelling DNA Probe.
Fragments of DNA were radiolabelled with $\alpha$-$[^{32}\text{P}]$-dCTP. The method is based on the random primer labelling reaction previously described (Feinberg and Vogelstein 1983). DNA was digested (section 2.1.4.2.) and run on a 1% TAE agarose gel (section 2.1.6.). The required DNA fragment was then excised from the gel and purified using a GeneClean II Gel Extraction Kit (Bio 101, Vista, CA, USA) as per manufacturer’s instructions. The DNA was eluted in a final volume of 20μl. Approximately 5μl of the purified DNA was then placed in a screw cap Eppendorf and heated to 100°C for 5mins and then snap cooled by placing on ice for 2 mins. Into this tube were placed 10μl of oligolabelling buffer, 5 units of DNA polymerase large fragment (Klenow), 50μCi of $\alpha$-$[^{32}\text{P}]$-dCTP. The volume was made up to 50μl with ddH₂O. The reaction was then incubated at 37°C for 30mins. The reaction was then filtered through a G50 Sephadex column to remove any unincorporated label. The radiolabelled DNA probe was then heated for 5mins at 100°C and snap cooled on ice for 2mins. The denatured probe was then added to the hybridisation solution.

**Oligolabelling Buffer (OLB):**

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

- **Solution A:** 1ml 1.25M Tris HCl (pH 8.0)/ 0.125M MgCl₂
  - 18μl β-mercaptoethanol
  - 5μl 0.1M dATP
  - 5μl 0.1M dGTP
  - 5μl 0.1M dTTP
- **Solution B:** 2M HEPES pH 6.6
- **Solution C:** 90 units/ml random hexamers [pd(N)₆]
  - Dissolved in TE pH 8.0
2.1.6. *Agarose Gel Electrophoresis.*

1% (w/v) agarose gels were made using 1x TAE (0.4M Tris, 0.2M sodium acetate, 20mM EDTA, pH 8.3). Ethidium bromide was added to a final concentration of 0.5µg/ml. Loading buffer (1x TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. A 1 kilobase DNA ladder (Gibco, UK) was used in all cases as a marker for DNA size.

2.1.7. *Southern Blot Analysis of Viral DNA.*

Southern blots were performed on viral DNA to determine that correct homologous recombination had occurred when producing recombinant HSV vectors.

2.1.7.1. *DNA Preparation.*

20µl of each viral DNA prep (section 2.1.3.3.) including a negative control, were digested overnight with the appropriate enzymes and buffers in a total volume of 50µl. 0.1µg of plasmid DNA (positive control) was digested in a total volume of 20µl. The digested reactions were then run on a 1% TAE agarose gel until DNA fragments were well separated. The DNA was visualised on a UV transilluminator and photographed with a fluorescent ruler.

2.1.7.2. *DNA Transfer (Southern Blot).*

The gel was left on the transilluminator for 2mins and for further denaturation was then placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 45mins. After rinsing briefly in H₂O the gel was then transferred to neutralising solution (2M NaCl, 1M Tris pH 5.5) for at least 30mins. The gel was then placed upside-down on a plastic support which was covered in a layer of 3mm Whatman paper (Whatman, Maidstone, UK), which was used as a wick placed in a reservoir of 20x SSC (150mM NaCl, 15mM sodium citrate pH 8.0). 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, where placed on top of the nylon membrane and a stack of dry paper towels was placed on top with a weight. The DNA was then transferred by capillary action to the nylon membrane overnight. The membrane was removed
and washed in 6x SSC and then cross-linked twice (UV Stratalinker 2400). The membrane was then air dried for 30mins. The membrane could then be stored at 4°C prior to hybridisation.

2.1.7.3. Membrane Analysis.
Southern blot membrane analysis was performed using a radiolabelled probe designed to distinguish recombinant vector from non-recombinant vector. The DNA probe was radiolabelled as previously (section 2.1.5.3.) and hybridisation was carried out as before (section 2.1.5.2.).

2.2. TISSUE CULTURE.
Tissue culture plasticware was obtained from Nunc, Roskilde, Denmark. All media and supplements were supplied by Gibco-BRL Life Technologies, Paisley, Renfrewshire, UK. All viral preparations were carried out under Health and Safety Category 2 Conditions.

2.2.1. Virus Strain.
All HSV-1 vectors described here were developed from the 17syn+ HSV-1 strain isolated in Glasgow (Brown et al. 1973). Vector 1764 (Coffin et al. 1996) refers to 17syn+ deleted for both copies of ICP34.5 (MacLean et al. 1991a) and deficient in the C-terminal transactivation domain of VP16 (Ace et al. 1989). The VP16 deficiency was complemented for by addition of hexamethylene bis-acetamide (HMBA) to the growth media, to a final concentration of 3mM.

2.2.2. Mammalian Cell Lines.

2.2.2.1. Media.
Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100units/ml penicillin/streptomycin is referred to as full growth media (FGM). Dulbecco’s modified Eagle medium (DMEM) supplemented with 100units/ml penicillin/streptomycin is referred to as serum free media (SFM).
2.2.2.2. Baby Hamster Kidney Cells.

Baby Hamster Kidney cells (BHKs) Clone 13 (Macpherson and Stoker 1962) were provided by the Imperial Cancer Research Fund (ICRF), London, UK. BHK cells were cultured in FGM. Cells were passaged by washing in Hanks Balanced Salt Solution (HBSS) at room temperature and then incubated with a minimal volume (enough to cover the area of flask) of 10% (v/v) trypsin in versene at 37°C/5% CO₂. An appropriate volume of FGM was added to neutralise the trypsin/versene and the cells were then divided accordingly.

2.2.2.3. 27/12/M:4 Cells (MAM49).

27/12/M:4 cells are a stable cell line derived from BHK cells and have previously been described (Thomas et al. 1999b). The cells complement mutations in HSV-1 genes ICP4, ICP27 and VP16. Cells were cultured in FGM containing 5% (v/v) tryptose phosphate broth, 800µg/ml of Neomycin (Gibco-BRL Life Technologies, Paisley, UK) and 750µg/ml of Zeocin (Cayla, Toulouse, France). Cells were passaged as for BHK cells (section 2.2.1.2.). When cells were grown for viral infection all antibiotics were omitted.

2.2.2.4. ND7 Cells.

ND7 cells were created by fusion of mouse neuroblastoma cells (N18Tg2) with a rat post-mitotic neonatal dorsal root ganglion neuron (Wood et al. 1990). Cells were grown in Leibovitz L15 media supplemented with 10% FCS, 100 units/ml of penicillin/streptomycin, 0.35% (w/v) glucose, 2mM L-glutamine and 0.375% (w/v) sodium bicarbonate. Cells were passaged by removal of media and addition of 10mls of fresh L15 supplemented media followed by gentle agitation of the flask to dislodge the cells. The suspended cells were then divided as required into fresh L15 supplemented media.

2.2.2.5. T-Rex™-HeLa Cells.

HeLa cells are a human cervical adenocarcinoma cell line (ATCC* CCL-2). T-Rex™-HeLa cells (Invitrogen, Groningen, The Netherlands) stably express the Tet repressor (Yao et al. 1998) from the pcDNA6/TR plasmid (Invitrogen, Groningen, The Netherlands). Cells were cultured in FGM supplemented with 2mM L-glutamine, containing 5µg/ml Blasticidin (Invitrogen, Groningen, The Netherlands).
Netherlands). Cells were cultured and passaged as for BHK cells (section 2.1.2.2.).

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

2.2.2.6 Vero Cells.
Vero cells are an African green monkey kidney cell line (ATCC CCL81). Cells were grown in FGM and passaged as for BHK cells (section 2.2.2.2.)

2.2.3. Cell Line Storage.
Cells were trypsinised (section 2.2.2.2.) and spun at 1500rpm for 10mins to pellet. The cells were then resuspended in freezing media A (DMEM supplemented with 20% FCS) followed by addition of an equal volume of freezing media B (DMEM supplemented with 40% FCS and 16% dimethlysulphoxide [DMSO]). For an 80cm² tissue culture flask cells would be resuspended in a total volume of 9ml. Cells were aliquoted into 1.5ml cryovials and were maintained at −70°C for 24hours prior to storage in liquid nitrogen. Frozen cell stocks were rapidly thawed at 37°C and transferred immediately to 10ml of fresh FGM and pelleted by centrifugation at 1500rpm for 10mins. The cell pellet was then resuspended in 0.5ml of fresh FGM and titrated by 1:2 dilutions in 8 wells of a 24 well dish. Wells were then harvested and the cells transferred to flasks.

2.2.4. Transient Transfections Assays.

2.2.4.1. DNA Transfections.
Transfections were performed with plasmid DNA prepared by the midi-prep method (section 2.1.3.2.). The transfections were performed based on the calcium phosphate-mediated method as previously described (Stow and Wilkie 1976). Cells were grown in 35mm wells until they were 80% confluent. Two tubes were set up labelled A and B. Tube A contained 31μl 2M CaCl₂, 10μg plasmid DNA and 20μg herring sperm DNA (phenol/chloroform extracted). Tube B contained 400μl HEBES transfection buffer.
HEBES Transfection Buffer: 140mM NaCl
5mM KCl
0.7mM Na₂HPO₄
5.5mM D-glucose
20mM Hepes, pH 7.05 with NaOH

Filter sterilised
with a 0.2μm
filter.
Stored at 4°C.

The contents of tube A were carefully mixed by gentle pipetting and then added to tube B drop-wise with gently mixing. The mixture was then left for 20-40mins to allow the DNA to precipitate. Media was removed from the cell monolayer and the precipitated DNA mixture was then added (one transfection to each 35mm well) and incubated for 4hrs prior to DMSO shocking. To DMSO shock, media was removed from the cells and the cells washed twice with 2mls of FGM. 1ml of ice cold 25% (v/v) DMSO in HEBES transfection buffer was added to the cells and left for 1.5mins. The DMSO solution was removed and the cells washed twice with 2mls of FGM. A final 2mls of FGM was added to the cells and then they were incubated at 37°C/5% CO₂.

2.2.4.2. β-Galactosidase Activity Assay.

The level of activity of β-galactosidase was determined using the Galacto-Light™ β-galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA). This kit is a chemiluminescent reporter assay that allows the rapid, sensitive and non-isotopic detection of β-galactosidase activity. The transfected cells were washed in 1x PBS (10x Stock PBS: 104mM sodium chloride, 1.8mM potassium chloride, 5.4mM disodium orthophosphate dihydrate, 1.25mM potassium dihydrogen orthophosphate, pH 7.0) and then 200μl of lysis buffer was added, as per manufacture’s instructions. The cells were then harvested by scraping with a 1ml-syringe barrel. The cell debris was then pelleted at 2000rpm and the supernatent removed and stored at -80°C until needed. 1-20μl of cell lysate was assayed in triplicate using a Turner TH-20e luminometer as per manufacturer’s instruction. Occasionally the lysate required a 1:10 dilution in lysis solution before assay.
2.2.4.3. Detection of β-Galactosidase by X-Gal Staining.

Media was removed from the cells and then washed twice with 2mls of 1x PBS. The cells were then fixed with 1ml of 1x PBS containing 0.05% glutaraldehyde for 10mins. The cells were then washed twice with 2mls of 1x PBS before the addition of 2mls of X-Gal solution (5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆.6H₂O, 1mM MgCl₂, and 150µg X-Gal - dissolved in DMSO in 1x PBS). The cells were then incubated overnight. The X-Gal stain was then removed and the cells were washed twice in 1x PBS followed by addition of 2mls of 70% v/v glycerol. The cells were then stored at 4°C until photographed.

2.2.5. Production of Recombinant Replication Competent HSV-1 Vectors.

3mM Hexamethylene bisacetamide (HMBA) was included in FGM for the propagation and growth of all viruses containing the in1814 mutation (Ace et al. 1989). BHK cells were used in all cases.

2.2.5.1. Homologous Recombination Transfections.

Transfections were carried out as described in section 2.2.4.1. except 10-30µg of purified viral DNA (see section 2.1.3.4.) was added to tube A and the transfection was left for 7hrs before DMSO shock. The 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 (see section 2.2.5.2.) and the efficiency of the recombination determined by assaying for recombinants containing the required reporter gene.

2.2.5.2. Viral Titre Assay.

Cells were grown in 35mm plates until 80% confluent. A 1:10 serial dilution of virus suspension (either harvested from homologous recombination transfections or from pure stock) from 1x10⁰ – 1x10⁷ml was added to 0.5mls of serum free media and placed on the cells. The cells were then incubated for 1hr at 37°C/5%CO₂. The media was then removed and replaced with 2mls of 1:2 of 1.6% (v/v) carboxymethyl cellulose(CMC):FGM supplemented with 3mM HMBA if necessary. The cells were then incubated for a further 48hrs at 37°C/5%CO₂ and the wells were then assayed for number of plaques in each well in order to determine titre of virus. If the reporter gene was β-gal then viral titre was
determined following X-Gal staining (section 2.2.4.3.). If GFP was the reporter gene then plaques were observed directly under an inverted fluorescent microscope at a wavelength of 520nm. If the virus contained no reporter gene, the cells were fixed (see section 2.2.4.3) and 1ml of a 0.05% w/v crystal violet solution (in 20% EtOH) was added to the cells for 1min in order to visualise the plaques. The titre of virus was measured in plaque forming units per ml (pfu/ml).

**2.2.5.3. Purification of Viral Recombinants by Plaque Selection.**

Harvested wells from homologous recombination transfections were titred out as described in section 2.2.5.2. except that cells were not fixed. After X-gal staining (see section 2.2.4.3.) or UV light visualisation, appropriate plaques were picked from the cell monolayer using a P20 Gilson micropipette (set at 3μl) underneath an inverted microscope/fluorescent microscope. Plaques containing the required recombinant vector were isolated and transferred to a vial containing 100μl of SFM. The plaque suspension was then freeze-thawed. 10μl and 90μl of the plaque suspension were then added to 0.5ml of SFM that was then placed into a 35mm dish containing cells at 90% confluency. The cells were then incubated for 1hr at 37°C/5%CO₂. The media was then removed and replaced with 2mls of 1:2 of 1.6% (v/v) carboxymethyl cellulose: FGM supplemented with 3mM HMBA. The cells were then incubated for 48hrs. The plaque purification process was repeated until a pure population of recombinant vector was obtained. When a pure recombinant population was observed a single plaque was used to infect one well of a 35mm plate and left for 3days before harvesting. This was know as the master stock (MS) of virus and was stored at -80°C. Determination of viral titre in the MS was performed as described in section 2.2.5.2.

**2.2.5.4. Production of High Titre Stock of Recombinant Vector.**

The MS suspension was used to infect 2 x 175cm² flasks, containing BHK cells at 90% confluency, at a multiplicity of infection (MOI) of between 0.1 and 0.01. Cells were infected in 5ml of SFM and left for 1hr. Cells were then overlaid with FGM containing HMBA if necessary. The cells were incubated at 37°C/5%CO₂ until complete CPE was observed. The cells were then harvested and the viral titre determined. This stock was termed the sub-master stock (SMS). 10 x
850cm² roller bottles (Corning Glass Works, Corning, New York, USA) containing BHK cells grown to 90% confluency and were infected with 2x10⁶ pfu per roller bottle of virus, in a total volume of 100mls of FGM per roller bottle supplemented with 3mM HMBA if necessary. The cells 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. The cellular debris was then pelleted by centrifugation at 3000rpm for 30mins at 4°C. The supernatants were then combined and the virus particles pelleted by centrifugation at 12000rpm for 2hrs at 4°C. The supernatant was then removed and 1-3mls of this supernatant was then used to resuspend the viral pellet by gentle pipetting. The resuspended viral pellet was then sonicated for 1min in a water bath sonicator. 100μl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was determined using the standard viral titre assay (see section 2.2.5.5.) except that 1:10 serial dilutions were made between 1x10⁻³-1x10⁹ mls.

2.2.6. Production of Recombinant Replication Incompetent HSV-1 Vectors.
3mM Hexamethylene bisacetamide (HMBA) was included in FGM for the propagation and growth of all viruses containing the in1814 mutation. (Ace et al. 1989). 27/12/M:4 cells were used at all stages.

2.2.6.1. Homologous Recombination Transfections.
Transfections were performed as in section 2.2.4.1 except that transfections were left between 5-7 days before they were harvested. Complete CPE was never observed and transfections were harvested when plaques could be clearly be seen in the monolayer.

2.2.6.2. Purification of Viral Recombinants by Plaque Selection.
Cells were grown on 35mm plates until 90% confluent. Media was removed and 300μl, 100μl and 10μl of harvested transfections were plated out onto each well and made up to 0.5ml with SFM. Cells were left for 1hr at 37°C/5%CO₂ and then overlayed with FGM containing 3mM HMBA. Cells were left for 2days and then plaques were observed and picked following the methods described in section 2.2.4.3. except that no CMC was used. When a pure recombinant
population was observed a single plaque was used to infect one well of a 35mM plate and left for 3 days before harvesting. This was known as the master stock (MS) of virus.

### 2.2.6.3. Growing High Titre Stocks of Virus.

Approximately 300 μl (per well) of MS was used to infect 90% confluent 27/12/M:4 cells in 35mm dishes. The virus was made up to 0.5ml with SFM and the cells left for 1 hr at 37°C/5%CO₂. Cells were then overlaid with FGM containing HMBA if necessary. After complete CPE was observed cells were harvested and virus was then grown in increasing quantities in 80 cm² flasks and 175 cm² flasks until enough virus was obtained (see later). 27/12/M:4 cells were grown to 90% confluency in 10 x 224 mm² plates. Each plate was infected with 5 x 10^6 pfu of virus stock suspension in a total volume of 50mls of FGM supplemented with HMBA if necessary. Cells were harvested approximately 3 days later when complete CPE was observed. Cells were harvested using a sterile cell scraper (Nunc, Roskilde, Denmark) and freeze thawed before the suspension was spun at 3500 rpm for 45 mins to remove the cell debris. The supernatant was then sequentially filtered through a 5.0 μm filter (Whatman, Maidstone, UK) and a 0.45 μm filter (Gelman Life Sciences, Ann Arbor, Michigan, USA). To pellet the virus particles the supernatant was then spun at 12000 rpm for 2 hrs. The supernatant was then completely removed and the virus pellets were resuspended by gentle pipetting in a minimal volume of this supernatant. The pellets were combined in a total volume of ~500 μl or less. The resuspended viral pellet was then sonicated for 1 min in a water bath sonicator. 50 μl aliquots of the resuspended virus were stored in liquid nitrogen. The titre of the resuspended virus was determined using the standard viral titre assay (see section 2.2.5.2.) except that 1:10 serial dilutions were made between 1 x 10⁻³ - 1 x 10⁻⁹ mls.

### 2.2.7. Protein Extraction and Analysis.

If protein was to be extracted from virally infected cells, cells were harvested 24 hrs post-infection. If protein was to be extracted after transient transfection of plasmid DNA, cells were harvested 48 hrs post-transfection.
2.2.7.1. Standard Protein Extraction from Cultured Cells.
Cells were washed twice in 1x PBS and then harvested in a suitable volume of protein sample buffer (100μl per 35mm well [5% β-mercaptoethanol, 50mM Tris-HCl pH8.9, 6% (v/v) glycerol, 2% (w/v) SDS and 0.005% (w/v) bromophenol blue]). Samples were placed at 95°C for 5mins to denature the proteins and then left at -20°C until needed. Before loading onto an SDS-polyacrylamide gel samples were re-boiled at 95°C for 5mins and immediately put on ice.

2.2.7.2. Extraction of Multiple TM Spanning or Fragile Proteins from Cultured Cells.
Cells were washed twice with 1x PBS and then harvested in 1ml of PBS (per 35mm well). Cells were pelleted at 2000rpm for 3mins and the pellet resuspended in 100μl of 2x extraction buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 1mM PMSF, 10μg/ml leupeptin and 10μg/ml pepstatin). The resuspended cells were then passed 10x through a 21-gauge needle to shear the cells. Samples could then be stored at -20°C or further processed by adding 100μl of 2x TM protein sample buffer (4.8% (w/v) SDS, 2M urea, 8% (w/v) sucrose, 350mM β-mercaptoethanol). The samples were then stored at -20°C. Before loading onto an SDS-polyacrylamide gel samples were incubated at 37°C for 15mins. When samples were prepared by this method both resolving and stacking gels contained a final concentration of 2M urea (see section 2.2.7.3.)
2.2.7.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).
Approximately 10μl of sample and 5μl of coloured molecular weight markers were loaded per lane. SDS-polyacrylamide gels were prepared and run in a vertical gel electrophoresis system according to the method of (Laemmli 1970). The composition of stacking and resolving gels can be seen in table 2.1. All resolving gels used were 10%. Gels were run at a constant current (30-40mA/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 was approximately in the middle of the gel, as determined by the migration of the coloured molecular weight markers.

<table>
<thead>
<tr>
<th>Resolving gel: 30ml of 10% gel</th>
<th>Stacking gel: 10ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>H₂O</td>
</tr>
<tr>
<td>30% acrylamide mix*</td>
<td>6.8ml</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>30% acrylamide mix*</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10.0ml</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>7.5ml</td>
</tr>
<tr>
<td>0.3ml</td>
<td>1.0M Tris (pH 6.8)</td>
</tr>
<tr>
<td>0.3ml</td>
<td>12.5ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10% SDS</td>
</tr>
<tr>
<td>12μl</td>
<td>0.3ml</td>
</tr>
<tr>
<td></td>
<td>10% ammonium persulfate</td>
</tr>
<tr>
<td></td>
<td>0.1ml</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
</tr>
<tr>
<td></td>
<td>10μl</td>
</tr>
</tbody>
</table>

Table 2.1. SDS-PAGE gel composition. *Acrylamide mix = acrylamide-bis (30:1.5). TEMED = NNNN-tetrethylethalaminediamine

2.2.7.4. Transfer of Proteins to Nitrocellulose Membranes (Western Blot).
Proteins were transferred from the SDS-polyacrylamide gel to Hybond C nitrocellulose membranes by a wet transfer method, based on that previously described (Towbin et al. 1992). Briefly, the SDS-PAGE gel and the nitrocellulose membrane were pre-soaked in transfer buffer (50mM Tris, 180mM glycine, 0.1% (w/v) SDS and 20% v/v methanol) and then sandwiched between sheets of 3mm Whatman paper (Whatman International Ltd, Maidstone, Kent) and a Trans-Blot™ cell (Bio-rad Hemel Hempstead, Herts) assembled according to the manufacturer’s instructions. Transfer was carried out either overnight at 20mA and 4°C or over 2hrs at 70mA and 4°C.
2.2.7.5. Immunodetection of Proteins on Western Blots.

Proteins were detected on western blots following the method detailed below (table 2.2.). Throughout the protocol the blots were shaken constantly at RT. Dilutions and details of primary antibodies can be seen in table 2.3. All Secondary antibodies were anti-IgG horseradish peroxidase (HRP) conjugated.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Block</td>
<td>5% (w/v) skimmed milk in 1x PBS</td>
<td>1hr</td>
</tr>
<tr>
<td>2. Primary Antibody</td>
<td>Diluted in 3% (w/v) skimmed milk in 1x PBS</td>
<td>1-2hrs</td>
</tr>
<tr>
<td>3. Wash</td>
<td>1x PBS, 0.1% Tween-20 (1x PBST)</td>
<td>3x 10mins</td>
</tr>
<tr>
<td>4. Secondary Antibody</td>
<td>Diluted 1:1000 in 3% (w/v) skimmed milk in 1x PBS</td>
<td>1hr</td>
</tr>
<tr>
<td>5. Wash</td>
<td>1x PBS, 0.1% Tween-20 (1x PBST)</td>
<td>3x 10mins</td>
</tr>
</tbody>
</table>

Table 2.2. Western blot protein detection protocol.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre recombinase</td>
<td>Monoclonal anti-mouse IgG</td>
<td>BAbCO Richmond, California, USA.</td>
<td>1:500</td>
</tr>
<tr>
<td>Retinoid-X-Receptor RXRα</td>
<td>Polyclonal anti-rabbit IgG</td>
<td>(D-20) Santa –Cruz Biotechnology, Santa-Cruz, California.</td>
<td>1:1000</td>
</tr>
<tr>
<td>Tetracycline Repressor</td>
<td>Monoclonal anti-mouse IgG</td>
<td>BD-Clontech Basingstoke, UK</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 2.3. Primary antibody details.

2.2.7.6. Equalisation of Protein Loading in Western Blot Analysis.

Duplicate protein samples were separated by SDS-PAGE 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 1hr at room temperature with continual shaking. Any unbound stain was then removed by repeated replacement of destain solution (10% (w/v) glacial acetic acid, 30% (v/v) methanol).

2.2.8. In Vivo Vector Delivery.

3-week-old female BALB/c mice (Harlan Laboratories, Oxon, UK.) were used in all experiments except where described.
2.2.8.1. Footpad Inoculation.
Mice were lightly anaesthetised by fluothane inhalation and inoculated in the left rear footpad, as previously described (Coffin et al. 1996), with 25μl of a stock of recombinant vector (inoculum titres ranged from $1 \times 10^8$ to $5 \times 10^8$ pfu/ml). At various times post-inoculation the mice were sacrificed and the ipsilateral lumbar dorsal root ganglia (DRG) L1-L6 were removed and reporter gene expression determined (sections 2.2.8.3. and 2.2.8.5.).

2.2.8.2. Sciatic Nerve Inoculation.
Animals were anaesthetised by fluothane inhalation for as long as necessary. A skin incision was made over the left gluteal muscles and the sciatic nerve exposed following blunt dissection of overlying muscle. The sciatic nerve was placed under tension and 2-5μl of a stock of recombinant vector was injected using a glass micropipette (inoculum titres ranged from $1 \times 10^7$ to $5 \times 10^8$ pfu/ml). Tension was then released, the wound was closed and animals allowed to recover. DRG were then removed at various time points post-inoculation as for the footpad inoculation.

2.2.8.3. Detection of β-Galactosidase Activity in Extracted Dorsal Root Ganglia.
Dorsal root ganglia (DRG) were dissected from the mice and placed in 1 x PBS. The DRG were then fixed with 4% paraformaldehyde in 1 x PBS for 1hr on ice. The DRG were then washed 3 times with 1x PBS for 15mins each wash. The DRG were then placed in 100μl of DRG X-Gal solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6\cdot6H_2O$, 1mM MgCl$_2$, 0.02% sodium deoxycholate, 0.02% NP-40, and 40mg/ml X-Gal [dissolved in DMSO] in 1x PBS) and incubated at 37°C overnight. The X-Gal solution was then removed and the DRG placed in 70% v/v glycerol and stored at 4°C prior to photography. DRG were photographed by placing in 1 x PBS on a glass slide and placing a coverslip on top. Photographs were taken at either x5 or x10 magnification on a Zeiss Axiophot microscope.
2.2.8.4. Detection of Green Fluorescent Protein in Extracted Dorsal Root Ganglia.

DRG were removed and placed in 1x PBS. The DRG were then placed on a glass slide using a plastic pipette tip and coverslipped. GFP expression was then visualised under UV light (520nm) under either x5 or x10 magnification and photographed. When dual expression of GFP and \textit{lacZ} was to be assessed, GFP fluorescence was examined prior to X-Gal staining as otherwise LacZ masked the GFP fluorescence.

2.2.8.5. Counterstaining of Sectionned Animal Tissue.

Sectionned DRG tissue was counterstained with either thionin or neutral red.

- Thionin (Sigma, Dorset, UK)

A 1% v/v thionin solution in 0.1M acetic acid and 0.1M sodium acetate was diluted to 0.1% in 0.1M acetic acid and 0.1M sodium acetate, pH 4.2. The solution was filtered before use and used immediately. Slides were dipped in stain for 2mins and then rinsed in distilled water.

- Neutral red

5ml of 1% (w/v in dH$_2$O) neutral red was added to 1ml of 1M NaOAc (pH 8.5) and 1ml of 1.2M acetic acid (pH 2.4) and made up to 250ml in dH$_2$O. Slides were placed in this solution for at least 30mins.

After counterstaining, the slides were dehydrated by dipping sequentially into 70% EtOH, 95% EtOH, and absolute EtOH for 10mins each. The sections were then cleared using Histoclear (National Diagnostics) for 30mins. The sections were then drained of Histoclear and the sections mounted with glass coverslips using DePeX mounting medium (BDH).
CHAPTER 3:

IDENTIFICATION OF OPTIMAL REPLICATION COMPETENT VECTORS FOR GENE DELIVERY TO THE PNS
CHAPTER 3:
IDENTIFICATION OF OPTIMAL REPLICATION COMPETENT VECTORS FOR GENE DELIVERY TO THE PNS.

3.0. INTRODUCTION.
Many disease pathologies mean that effective treatment will require long-term or even lifelong gene expression. Thus the initial aim of this work was to identify a non-pathogenic HSV-1 vector for long term transgene delivery to the peripheral nervous system (PNS). Since the HSV lifecycle involves infection of peripheral nerves, vectors based on HSV would seem the natural choice for development of gene therapy in the PNS. There have been a number of previous studies using HSV-1 vectors for gene delivery to the PNS and some of these will be discussed.

The earliest vectors described were deleted for the non-essential gene tk and contained expression cassettes employing HSV promoters such as the tk or ICP4 promoter (Ho and Mocarski 1988;Palella et al. 1988). Whilst these vectors achieved short-term gene expression in vitro and in vivo, no gene expression was seen in the long term. In another study vectors mutated for VP16 showed either no expression, or low levels of gene expression during latent infection compared to the levels observed during acute infection (Ecob-Prince et al. 1995). However, the viruses tested were all shown to establish latency efficiently. Other groups have shown that vectors containing insertions in the gC locus have also given efficient gene delivery but again only in the short term (Lokensgard et al. 1994;Dobson et al. 1995). Using an alternative gene deletion strategy and one that has been successfully developed in this thesis, our group has previously shown that vectors deleted for ICP34.5 or ICP34.5 and an inactivating mutation in VP16 show efficient gene delivery to peripheral ganglia (Coffin et al. 1996). However, again this was only achieved in the short term.

The vectors discussed above have all retained some degree of replication competence both in vivo and in vitro, inasmuch as none of their essential genes have been removed. In addition, they all expressed reasonable levels of transgene during acute infection, which reduced considerably during latency.
even though a latent infection was established. Thus, the initial focus of this thesis was to further define parameters and possible gene deletions that would allow replication competent vectors to give efficient gene delivery to spinal ganglia. It was initially hypothesised that some level of replication competency would be required for gene delivery following peripheral administration. Reasons for this were that the HSV-1 lifecycle might be anticipated to require peripheral replication in epithelial cells before uptake by sensory nerve terminals, and reports of replication incompetent viruses allowing gene delivery to the PNS have been limited (discussed in chapter 4). Thus, following footpad inoculation at least, it is possible that only vectors capable of in vivo replication would result in efficient gene delivery vectors.

A second aim was to identify promoters with the ability to retain transgene expression during latency, as most HSV and non-HSV promoters become inactivated during the latent period (Ho and Mocarski 1988; Palella et al. 1989; Chiocca et al. 1990b; Fink et al. 1992). Using replication competent vectors some groups have now achieved transgene expression in the PNS for up to 6 months, using the latency associated promoters to drive reporter gene expression. Insertions were made either directly into the LAT region using an internal ribosome entry site (IRES) in front of the lacZ gene (Lachmann and Efstathiou 1997a). Alternatively expression cassettes were inserted into the gC locus (Berthomme et al. 2000; Goins et al. 1994; Lokensgard et al. 1997), indicating that the LAT promoters can function at ectopic sites in the HSV-1 genome. These later vectors contained either a LAP2-lacZ construct and gave very weak latent expression (Goins et al. 1994), or a LAP1-LTE/lacZ construct (Lokensgard et al. 1997; Berthomme et al. 2000). The LTE sequence is a 3' extension of the LAP2 promoter previously described (Goins et al. 1994). Goins et al. suggested that a LAP2-lacZ/gC vector could produce reporter gene expression up to 300 days post inoculation. However, this has not been shown either by detection of lacZ transcripts, or by histochemical staining. In addition, and in support of results obtained from constructs described in this thesis, it was shown that the long-term element (LTE) can function in a bi-directional manner to confer long term activity on LAP1 (Lokensgard et al. 1997; Berthomme et al. 2000).
Not only have the LAT promoter elements shown promise for latent transgene expression in the PNS, but the Moloney murine leukemia virus-LTR has shown previous potential. It was found that the MMLV-LTR was capable of directing latent transgene expression when juxtaposed to the LAP1 promoter and inserted into the gC locus (Lokensgard et al. 1994). However in the same report it was shown that LAP1 or MMLV alone, or a number of other promoters alone or linked to LAP1 did not give long-term transgene expression. The MMLVLTR alone, inserted in ICP4 (Dobson et al. 1990) or 5' to LAP1 (Carpenter and Stevens 1996) was shown to be active during latency, possibly due to the close proximity of these regions to the endogenous LAT region. These results highlight that MMLVLTR in conjunction with LAP1, like LAP1 linked to LAP2, can support latent transgene expression. In a different study, a vector mutated for VP16 and containing various promoter/reporter constructs (HCMV, MMLVLTR, IE1 and VP16) in the tk or UL43 loci, gave β-galactosidase activity during latent infection only from a HCMV promoter, but only at very low levels compared to those observed initially (Ecob-Prince et al. 1995).

The aim of this chapter was to test HSV-1 vectors for their ability to reach peripheral ganglia following peripheral administration, either via footpad injection or direct sciatic nerve injection. Viruses were tested that were serially deleted for both essential and non-essential genes (see chapter 4), to further assess the optimal combinations and the factors required for efficient gene delivery following peripheral administration. Vectors tested were all based on the 1764 backbone (HSV-1 stain 17+ (Brown et al. 1973)) that was deleted for both copies of ICP34.5 (MacLean et al. 1991a) and had an inactivating mutation in VP16 (Ace et al. 1989) (see figure 3.1). 1764 had previously been shown to allow transgene expression in the DRG following peripheral administration (Coffin et al. 1996). In addition to this, a second aim of this thesis was to identify promoter/reporter expression cassettes that allowed transgene expression at latent times after HSV infection.
3.1. MATERIALS AND METHODS.

3.1.1. Cyclosporin Injection.
Two days prior to virus inoculation animals were pre-treated with cyclosporin (Neoral: Sandoz, Basel Switzerland). Animals were anaesthetised and administered unilaterally with 100μl of a 50mg/ml stock of cyclosporin (5mg), diluted in castor oil, via the intraperitoneal (IP) route. Two days post virus inoculation cyclosporin was re-administered as before and this continued every two days over a 1 month period. Immunosuppressed animals were then sacrificed and DRG removed for analysis.

3.1.2. Fluororuby Injection.
Mice were anaesthetised and injected in the left rear footpad with increasing volumes (2.5μl, 5μl, 7.5μl, 10μl) of 10% Fluororuby in 1xPBS +0.2%DMSO (10000MW Fluororuby: Molecular Probes, Eugene, Oregon, USA) made up to 25μl with 1x PBS. 1 week post inoculation mice were sacrificed by cardiac perfusion with 4% paraformaldehyde (PFA [4% PFA in 1x PBS]) under terminal anaesthesia. Ipsilateral DRG and sciatic nerve were dissected and post fixed in 4% PFA for 1 hr and washed twice for 15 mins in 1x PBS. Tissue was placed on a slide in 1X PBS and a coverslip placed over the top. Observation of Fluororuby was performed using a rhodamine filter at 590nm.

3.1.3. In Situ Hybridisation.

3.1.3.1. Poly-L-lysine Coating Microscope Slides.
Twin frosted microscope slides (Chance Propper Ltd., UK) were wrapped in aluminium foil and baked at 180°C for 4hrs. Slides were then dipped in a 0.01% (w/v) poly-L-lysine solution (25mg of poly-L-lysine [MW 350000 Sigma, UK] dissolved in 5ml of Diethylpyrocarbonate [DEPC]-treated water and stored at 4°C. 1ml of this solution was diluted in 50ml of DEPC-treated water to make a 0.01% w/v solution). Slides were allowed to stand in racks and dry at room temperature and were then stored at 4°C in sealed containers with silica gel for 1-2 months.
3.1.3.2. Tissue Sectioning.
Animals were sacrificed at either 3 days or 1 month p.i. and DRG removed and snap frozen by placing on aluminium foil sitting directly on dry ice. DRG were wrapped in aluminium foil and stored at -70°C until sectioned. Without defrosting, tissue was mounted in OCT embedding compound (Histological Equipment Ltd. Nottingham, UK) and sectioned at 12μm using a cryostat (Leica, Nussloch, Germany). The tissue was mounted directly onto poly-L-lysine coated slides (see section 3.1.3.1.) and allowed to air-dry before fixation.

3.1.3.3. Fixation of Sections.
Racks of slides with air-dried tissue sections were fixed in 4% (w/v) PFA (in 1x PBS) for 5 mins at 4°C. Racks were rinsed in 1x PBS for 1 min and then transferred to 70% EtOH (in DEPC-treated water) for 5 mins. Slides were then placed in 95% EtOH (in DEPC-treated water) and stored in sealed containers at 4°C until necessary.

3.1.3.4. Labelling Oligonucleotide Probes.
In all cases a LAT specific oligonucleotide probe was used complementary to HSV-1 nt 120691 to 120647.
(5'-TAAAGGTTCATTGATCTTTTCCCTGTCTGTGTTGGATGTAT-3')

A reaction mixture was set up as follows:

5x tailing buffer \(^1\) 2.5μl
DEPC-treated water 4.5μl
Oligonucleotide (5ng/μl) 2.0μl
Terminal deoxynucleotidyl transferase TdT (Promega) 1.5μl
\(\alpha^{35}\text{S-dATP (Dupont NEN)}\) 1.5μl
\[\text{Total: 12μl}\]

The mixture was spun to the bottom of an Eppendorf tube and incubated for 1 hr at 37°C. The reaction was stopped by adding 38μl of DEPC water. Whilst the probe was labelling a spin column was prepared. The plunger from a sterile 1ml syringe was removed. The bottom of the syringe was blocked with autoclaved
silanised glass wool and packed down with a plunger. The column was filled with G-25 Sephadex (Pharmacia, St Albans, UK) made up with TNES (20mM Tris, 0.14mM NaCl, 5mM EDTA, 0.1% SDS made in DEPC-treated water). The column was pre-spun at 2000rpm for 1min to pack down the Sephadex (after which the column should contain at least 0.8ml of Sephadex). The column was then loaded with 50μl of the labelling reaction and re-spun at 2000rpm for 1min. The probe was recovered in a 1.5ml Eppendorf tube with the lid cut off. The probe was then analysed for the amount of α-35S-dATP incorporated. 2μl of probe was added to 4ml of scintillation fluid (Zinser Analytic) in a scintillation vial. The vial was shaken thoroughly and counted on a Beckman LS 1808 counter for 1min. The count should be around 200000dpm/μl of probe and if significantly lower a new preparation was made. 2μl of 1M dithiothreitol (DTT) was added to the probe in order to stop the formation of cysteine-cysteine disulphide bridges. The probe was stored at −20°C until use and was viable for at least a month (the half-life of α-35S-dATP is 3months).

1 Tailing buffer containing co-factor for TdT enzyme, supplied with the enzyme.
2 Silanised glass wool. A beaker was filled with glass wool and covered in 5% w/v dimethyl dichlorosilane made up in chloroform for 10mins. The wool was then washed copiously in DEPC-treated water and put in a warm oven to dry. All steps were carried out in a fume hood.

3.1.3.5. Preparation of Hybridisation Buffer.
50ml of hybridisation buffer was prepared as follows and stored at 4°C;
25ml of 100% deionised formamide
10ml of 20X SSC
2.5ml of 0.5M NaPO₄ pH7.0
0.5ml of 50X Denhardt’s Solution
2.5ml of 4ml/ml acid-alkali hydrolysed salmon sperm DNA
1ml of 5mg/ml polyadenylic acid [5’] (Sigma; in DEPC-treated water)
50μl of 120mg/ml heparin (in DEPC-treated water)
5g of dextran sulphate
Made up to 50ml with DEPC-treated water.
5g of monobed amberlite resin MB-1 were added to 50ml formamide (Fluka) and stirred for 30mins. The solution was filtered and stored at −20°C.

20X SSC; 3MNaCl, 0.3M sodium Citrate, pH7.0 with HCl. Made up to 1litre with dH2O, DEPC treated and autoclaved.

50X Denhardt’s; 1% (w/v) polyvinylpyrolidone (PVP), 1% (w/v) bovine serum albumin (BSA), 1% (w/v) Ficoll 400 in DEPC treated water. Stored at 4°C.

15ml of DEPC-treated water was added to 1mg of salmon sperm DNA and allowed to soak for 1hr. 2.5ml of 2M HCl was added and the white ball of DNA was gathered with a pipette tip and then added to 0.5ml of 2M NaOH. This was placed at 50°C in a water bath for 15mins or until the DNA dissolved. The DNA was then diluted to 175ml in DEPC-treated water and 20ml of 1M Tris pH7.4 was added. The pH was then adjusted to 7.5. The solution was filtered and stored at −20°C. The DNA concentration was then measured using a spectrophotometer at an OD of 260nm.

Dextran sulphate (Pharmacia) takes several hours to dissolve and thus the buffer was placed on a shaker overnight at 4°C.

3.1.3.6. Hybridisation of Probe to Tissue Sections.

Sections were removed from 95% EtOH and allowed to air dry for at least 30mins. The slides were placed in a bioassay dish (NUNC, Roskilde, Denmark) and labelled. The hybridisation buffer was pre-warmed at 42°C and vigorously shaken to remove any precipitate. 250000dpm of probe was dissolved in 100μl of buffer per slide and the mixture vortexed. 20μl of 1M DDT per ml of solution was then added and the buffer vortexed. The solution was then applied to each slide and each one was then gently coverslipped using a piece of parafilm cut to the correct size. For each probe/virus a competition control was set up as follows. The radiolabelled probe was prepared as above but 100-fold excess of unlabelled probe directly from the 5ng/μl stock oligonucleotide was also added. A piece of tissue saturated in 1x SSC was placed in the bioassay dish to create a humid atmosphere and a lid placed on the dish and an airtight seal made using parafilm. The dish was then placed at 42°C overnight.
3.1.3.7. Washing the Sections after Hybridisation.
After overnight hybridisation (section 3.1.3.6) slides were transferred to glass racks and dipped in prewarmed 1x SSC at 55°C. The parafilm was carefully removed. The slides were then washed twice for 30mins with gentle shaking at 55°C in 1x SSC. The slides were then washed in 0.1x SSC at RT for 1min, washed in 70% EtOH for 1min and washed in 95% EtOH for 1min. The slides were allowed to air dry for 30mins.

3.1.3.8. Emulsion Dipping In Situ Hybridised Sections.
This procedure was carried out in the dark under safe-lights.

15ml of 0.5% glycerol was warmed to 42°C. Emulsion (K5, Ilford, UK) was added so that the final volume was 30ml, making a ratio of 1:1 emulsion to solution. This mixture was left to melt for 30mins at 42°C and was then gently inverted to give it a thorough mixing. The emulsion solution was then placed in a dipping chamber and put securely at 42°C. The slides were individually dipped into the emulsion solution and the excess drained off. The slides were then left to dry in a humid atmosphere for 3-4hrs. The slides were then put in a light tight box containing silica gel wrapped in tissue paper and sealed with insulation tape and stored at 4°C until ready for developing.

3.1.3.9. Developing the Slides.
After a period of between 2-3 weeks the slides were ready for developing. The slides were transferred to glass racks under safe light conditions. The slides were immersed in Kodak D19 developer at 22°C for 2mins. (Prior to this the D19 was heated slightly until it dissolved [not above 40°C] and then cooled to 22°C with ice in a water bath). The slides were then rinsed briefly in distilled water and transferred to Ilford Hypam fixer for 5mins. The slides were then washed in dH2O for 10mins. The sections were counterstained as previously detailed (section 2.2.8.5.).
3.2. RESULTS.

3.2.1. LAP1 and LATP2.
Throughout this thesis LATP2 (identified in our laboratory) refers to a ~1.4 kb fragment downstream to the LAP1 transcriptional start site (HSV-1 nt 118866 to 120219 [PstI to BstXI]). This is a downstream extension of the LAP2 region identified by Goins et al (Goins et al. 1994). LAP1 refers to the TATA box containing promoter as named by Goins et al (Goins et al. 1994) and in this thesis refers to either HSV-1 nt 118443 to 118878 (NotI to StyI) or 118181 to 118878 (Ddel to StyI). See figures 3.1 and 3.3.

3.2.2. Single Transgene Expression from a Reporter Cassette in the LAT Region.
Previous work performed in our laboratory identified the MMLV-LTR promoter as capable of driving long term transgene expression, when linked to the LAP1 promoter and inserted into the UL43 locus (Palmer et al. 2000). It was found that this activity was enhanced when the two elements were separated by ~700bp of plasmid DNA (derived from pGem3Zf: Promega) rather than by a direct LAP1/MMLV-LTR fusion. This was similar to the work of Lokensgard et al (Lokensgard et al. 1994) who reported a comparable cassette inserted into the gC locus.

Based on the aforementioned information it seemed appropriate to compare the ability of the MMLV-LTR and CMV promoters to drive latent gene expression from a position directly in the LAT region. CMV is known to be a promiscuous promoter. Positioning of these promoters downstream to LAP1 and LATP2 elements would allow the testing of the hypothesis that the LAT region was structurally different to the rest of the genome and thus would allow promoters usually inactive during latency to remain active by placement in this region. Other work has been performed whereby an internal ribosome entry site (IRES), but no additional promoter, was placed in the 2kb LAT. This allowed expression of a marker gene for at least 190 days (Lachmann and Efstathiou 1997b). Also, the insertion of the β-galactosidase gene directly into the LAT region, again with
no additional promoter, downstream to the LAT transcription start site has been tested, but this gave only short term expression (Ho and Mocarski 1989).

3.2.2.1. Expression Cassette and Vector Production.

Previously two promoter/reporter cassettes had been constructed, pR19MMLVLTRlacZ and pR19CMVlacZ (see figure 3.1) and have now been described (Palmer et al. 2000). pR19MMLVLTRlacZ contains MMLVLTR-lacZ downstream to LAP1 and LATP2 and pR19CMVlacZ contains CMV-lacZ downstream to LAP1 and LATP2. Briefly, lacZ was cloned from pCH110 (HindIII-BamHI; Pharmacia), MMLV from pJ4Q (Nhe1-KpnI; (Morgenstern and Land 1990)) and CMV from pcDNA3 (654bp; Invitrogen). The cassettes were cloned into the HSV-1 LAT region using a 3.5kb NotI fragment (HSV-1 nt 118443 to 122029) inserted into pGem5 (Promega), see figure 3.1. In each case the promoter/lacZ cassette was cloned between BstXI sites (HSV-1 nt 120219 to 120413). The viruses were made separately by co-transfection of the plasmids with 1764 viral DNA (see protocol 2.2.5.) and purification of blue plaques from white. Plasmids were linearised using XmnI (pR19CMVlacZ) and MluI (pR19MMLVLTRlacZ). Due to the diploid nature of the LAT region the cassettes were present twice per viral genome. Vector recombinants containing two copies of the cassettes were distinguished from those harbouring only one copy by their ability to quickly form dark blue plaques upon X-Gal staining (protocol 2.2.4.3), compared to plaques that went light blue (data not shown).
Figure 3.1. Schematic diagram of promoter constructs and vector backbone tested for gene expression characteristics. A. 1764 vector backbone, deficient in ICP34.5 and VP16 and showing insertion sites in the LAT regions. Promoter/reporter cassettes were cloned into the HSV LAT region in a 3.5kb NotI fragment cloned into pGemS (HSV-1 nt 118443 to 122027). B. pR19MMLVLTRlacZ and C. pR19CMVlacZ. In each case the promoter/LacZ cassette is cloned between BstXI sites (HSV-1 nt 120219 to 120413). Arrows represent orientation of promoters.
3.2.2.2. In Vivo Analysis.

1764pR19MMLVLTRlacZ and 1764pR19CMVlacZ were tested in vivo for their gene expression characteristics. In both cases vectors were administered via footpad and sciatic nerve inoculation routes and DRG removed from 3 mice at each of the times p.i., beginning during acute infection at 2 days p.i. and ending at various times during latent infection.

Previously it has been shown that a few days after virus enters sensory neurons no free virus can be detected within ganglia and latency has been established (Stevens and Cook 1971). Analysis of viral gene expression has shown that during latency lytic genes are switched off and that LATs are the only set of transcripts expressed (Stevens et al. 1987 and reviewed in Fraser et al. 1992; Wagner and Bloom 1997). This therefore can be used as a marker of the establishment of latency, beginning a few days after neuronal infection and which is maintained thereafter.

Results can be seen in figure 3.2. and show that in all cases latent transgene expression is observed. Following footpad inoculation, β-galactosidase expression occurs at similar levels between the two viruses tested. However after sciatic nerve inoculation, the CMV promoter appears to give increased β-galactosidase expression during acute infection, compared to footpad inoculation, and rapidly drops during latent infection, whereas the MMLV-LTR promoter gives expression in only a small number of cells during both acute and latent stages of infection. The high levels of lacZ expression seen with the pR19CMVlacZ vector was probably as a result of direct injection into the nerve, where substantially more axons could be targeted compared to footpad inoculation. The fact that only the pR19MMLVLTRlacZ vector gave very low levels of lacZ expression following sciatic nerve inoculation but not after footpad inoculation, would suggest that this is an as yet undefined promoter specific phenomenon that is only observed following sciatic nerve injection.
Figure 3.2. Replication competent vectors, 1764pR19MMLVlacZ and 1764pR19CMVlacZ tested for gene delivery to DRG during acute and latent infection. A. Footpad inoculation with 20µl of virus at 1x10^8 pfu/ml. B. Sciatic nerve inoculation with 2-5µl of virus at 1x10^7 pfu/ml. C. Sciatic nerve inoculation with 2-5µl of high titre virus (1x10^8 pfu/ml). MMLVLTR, Moloney murine leukemia virus long terminal repeat promoter. CMV, cytomegalovirus promoter. lacZ, β-galactosidase.
3.2.3. **Multiple Transgene Expression from a Cassette in a Non-Essential Locus.**

Many diseases might require the simultaneous delivery of two therapeutic genes as a means of treatment. This could be achieved by the use of two different vectors, each providing one transgene or by the insertion of two transgenes into the same vector. Previously in our laboratory promoter systems were constructed that allowed multiple gene delivery from a single expression cassette. These cassettes, pR20.5 and pR20.9, are shown in figure 3.3. It has been shown previously by others that elements found in the LAP2 region can confer long-term gene expression activity on the LAP1 promoter (Lokensgard et al. 1994; Lokensgard et al. 1997). In addition it was shown that the MMLV-LTR promoter can give long term expression in combination with elements from LAP1 (Lokensgard et al. 1994). The LATP2 region provides the central element of both the pR20.9 and pR20.5 cassettes and is flanked on either side by two different promoters facing in opposite orientations (MMLV-LTR and LAP1 or CMV and RSV). These cassettes were inserted into either the UL43 or US5 loci of the HSV strain 1764.

3.2.3.1. **Non-Essential Genes UL43 and US5.**

US5 is a non-essential gene found in the unique short (Uₜ) region of the HSV genome and is one of 14 Uₜ genes, 13 of which are non-essential (Rasty et al. 1997). As predicted by sequence analysis, it has recently been identified as encoding a surface glycoprotein (gJ) (Ghiasi et al. 1998). A virus deleted for the US5 gene was shown to be viable for growth in cell culture, as determined on Vero cells. (Weber et al. 1987). More recently, a lacZ-US5 insertion mutant has been shown to have a normal phenotype both in vivo and in vitro (Balan et al. 1994). Here the virus was shown to have normal particle to infectivity ratio in vitro and no phenotypic abnormalities were observed in BHK cells following high or low MOI infectivity. In vivo ear scarification of mice showed that US5 mutants had no effect on the ability of HSV to multiply at the inoculation site or on the ability to enter or multiply in the PNS or CNS.

UL43 is located in the unique long (UL) region of the HSV genome. The gene has no known function although it contains multiple hydrophobic stretches and
is predicted to be a membrane channel protein (Carter et al. 1996). A UL43-lacZ insertion mutant and a UL43 deletion mutant were both shown to have no effect on the virus phenotype compared to the parental virus 17syn+, both in vitro, by comparing growth phenotypes, and in vivo (MacLean et al. 1991b). In vivo the viruses were compared in the mouse ear model, by their ability to replicate in the periphery or spread to and replicate within the nervous system. The UL43.5 ORF maps antisense to UL43, but is also known to be dispensable for growth in culture (Maclean et al. 1991b; Ward et al. 1996) and is of unknown function.

These suggested that UL43 and US5 were suitable sites for the insertion of expression cassettes.
3.2.3.2. Expression Cassettes and Vector Production.

Previously the two expression cassettes pR20.5 and pR20.9 had been constructed and have now been described (see figure 3.3) (Palmer et al. 2000) (Thomas et al. 1999b). Briefly, each cassette contains a central LATP2 region (HSV nt 118866 to 120219) flanked by two promoters. The 20.5 cassette contains CMV (Invitrogen) driving E-GFP (Clontech) and RSV (pRCRSV-Invitrogen) driving $\text{lacZ}$ (pCH110- Pharmacia). The 20.9 cassette contains MMLVLTR (pJ4Ω (Morgenstern and Land 1990)) driving E-GFP and the LAP1 promoter (HSV nt 118181 to 118878) driving $\text{lacZ}$. The cassettes were cloned into UL43 flanking regions at the unique A/s/1 site (HSV nt 94911 [in a BamHI-EcoRI fragment HSV nt 91619-96751 in pGem2]) or the US5 locus at the unique Sacl site (HSV nt 137945 [in a BamHI- EcoRI fragment 136289 – 139328]). The viruses were produced by co-transfection of the plasmids with 1764 viral DNA (section 2.2.5) and purification of green and blue plaques from white plaques. Three viruses were made and purified, 1764pR20.9/UL43, 1764pR20.9/US5 and 1764pR20.5/UL43.

3.2.3.3. In Vitro Vector Analysis on Permissive Cells.

During the purification procedure it became apparent that the phenotypes of the pR20.9 viruses were different from that of the pR20.5 virus. During lytic replication on permissive BHK cells, both of the pR20.9 containing viruses showed only low levels of $\beta$-galactosidase activity upon X-Gal staining (driven by the LAP1 promoter). However, both pR20.9 containing viruses showed strong GFP expression (driven by MMLVLTR). The pR20.5 virus showed strong expression of both $\text{lacZ}$ and GFP. See figure 3.4. This phenotype of the pR20.9 viruses appeared to be an active repression of LAP1 activity because when the virus is grown on BHK cells in the presence of acyclovir, which halts viral replication, $\beta$-galactosidase activity increases. However, here levels do not reach that observed with the pR20.5 virus (data not shown). In addition transient transfection of the pR20.9 plasmids results in strong X-Gal staining, i.e. in the absence of all viral proteins. Similar results to this phenomenon have been observed previously (Goins et al. 1994;Lachmann and Efstathiou 1997b). Lachmann et al describe vectors with either an IRES $\beta$-Geo cassette or an IRES $\text{lacZ}$ cassette inserted 1.5kb downstream to the LAP1 TATA box. These
viruses did not express detectable $\textit{lacZ}$ upon lytic infection of Vero cells (with low levels only in a few cells), but $\textit{lacZ}$ was readily detectable \textit{in vivo}. Goins \textit{et al} investigated the phenomenon to a greater extent by creating two viruses with LAP2/$\beta$-gal insertions in the gC locus, one with the additional deletion of ICP4. The gC mutated virus showed no $\beta$-galactosidase expression in Vero cells but expressed $\textit{lacZ}$ \textit{in vivo}, whereas the ICP4 deleted virus expressed $\textit{lacZ}$ both \textit{in vitro} and \textit{in vivo}. Goins \textit{et al} suggested that in the gC-virus, the actions of the ICP4 or ICPO proteins on LAP2 during lytic infection were responsible for the loss of $\textit{lacZ}$ activity observed. It is already known that the ICP4 protein down-regulates LAP1 during productive infection, by binding to the ICP4 binding motif found in the cap site (Batchelor \textit{et al}. 1994). It is thus possible that the action of either the ICP4 or ICPO proteins functions to silence LAP1 in the pR20.9 cassette. Since this repression is only evident in the pR20.9 cassette and no other cassettes studied in this thesis, it is likely that sequences in the LAP1 region must participate in this replication-dependent repression.
Figure 3.3. Expression cassettes and replication competent vector backbone. A. 1764 vector backbone deficient in ICP34.5 and VP16 and showing insertion sites in B. UL43 and US5 insertion sites. C. pR20.9 D. pR20.5. Both cassettes are flanked by restrictions sites for the blunt cutting enzyme SrfI allowing excision and insertion into appropriate flanking regions. RSV, Rous sarcoma virus promoter. MMLVLTR, Moloney murine leukemia virus promoter. GFP, Green fluorescent protein. LacZ, β-galactosidase. CMV, cytomegalovirus promoter. LAP1 and LATP2, HSV latency associated promoters. Arrows represent orientation of promoters.
A. 1764pR20.9/US5

B. 1764pR20.9/UL43

C. 1764pR20.5/UL43

Figure 3.4. Plaque phenotype of three replication competent vectors. A. 1764pR20.9/US5  B. 1764pR20.9/UL43  C. 1764pR20.5/UL43. Each vector contains a cassette with a central LATP2 region (HSV nt 118866 to 120219). The pR20.9 cassette contains LAP1 (HSV nt 118181 to 118878) driving lacZ expression and the Moloney murine leukemia virus long terminal repeat (MMLVLTR) driving GFP expression. The pR20.5 cassette contains Rous sarcoma virus promoter (RSV) driving lacZ and the cytomegalovirus promoter (CMV) driving GFP. See section 3.2.3. and figure 3.3.
3.2.3.4 **In Vivo Gene Expression is Affected by Promoter Choice and Delivery Route.**

Vectors were tested for gene delivery characteristics to lumbar ganglia following peripheral administration. All three vectors, 1764 pR20.9/UL43, 1764 pR20.9/US5 and 1764 pR20.5/UL43 were tested following footpad inoculation and the DRG were removed from 3 mice each for each vector at time points of 3 days p.i. and 1 week p.i. and during latent infection at 2 months and 6 months p.i. Vectors 1764 pR20.9/UL43 and 1764 pR20.5/UL43 were tested following sciatic nerve inoculation and DRG removed from 3 mice each at 3 days p.i., 2 weeks p.i. and 2 months p.i. After extraction, DRG were observed by fluorescent microscopy for GFP expression and stained with X-Gal for \( \text{lacZ} \) expression (see figures 3.5 and 3.6).

Results show that different levels of gene expression are observed between the vectors after both footpad and sciatic nerve inoculation, but that in all cases latent transgene expression is observed.

Following footpad inoculation reporter gene expression declines over time in all cases. pR20.9 gave consistently more GFP and \( \text{lacZ} \) positive cells than did the pR20.5 cassette, especially during latent infection when very few positive cells from pR20.5 could be detected. The pR20.5 cassette gave no positive cells at 6 months p.i., whereas the pR20.9 cassette consistently gave positive cells at 6 months albeit at lower levels than previous time points. Insertion of the pR20.9 cassette into US5 or UL43 gave identical results following footpad inoculation, indicating that the insertion position had no bearing on promoter activity, at least at these loci.

Since the pR20.9 vectors gave similar results after footpad inoculation when inserted into either US5 or UL43 it was decided to test only the 1764pR20.9/UL43 and 1764pR20.5/UL43 vectors following sciatic nerve inoculation. These results can be seen in figure 3.6 and unexpected they highlight a large discrepancy in reporter gene activity between the two vectors. The pR20.5 virus gave very high initial levels of GFP and \( \text{lacZ} \) activity, which at longer time points (during latent infection) declined to levels similar to those
observed after footpad inoculation. The initial high levels of lacZ and GFP expression observed at 3days was probably as a result of direct injection into the nerve, similar to the pR19CMV/lacZ vector, where substantially more axons could be targeted compared to footpad inoculation. The pR20.9 vector however gave only low levels of GFP and lacZ activity at 3days-post sciatic nerve inoculation, which increased at 2weeks and 2months p.i. This result could reflect the observations made with the same virus during lytic replication on BHK cells (section 3.2.3.3.), where lacZ activity is very low. It would be hypothesised that following sciatic nerve inoculation there are higher levels of ICP4 and/or ICP0 in transduced cells, compared to footpad inoculation, and that these mediate a repression of the pR20.9 cassette at early time points. Another group has reported similar observations with an essentially wild type virus, with IRES/lacZ inserted after LAP2, that gave increasing amounts of lacZ activity during latent infection compared to acute infection (Lachmann and Efstathiou 1997b). The same group reported that their virus did not express lacZ in vitro on Vero cells (as discussed earlier).

These results collectively suggest that gene expression during latency is a function of elements other than those located in the LATP2 region, since both the 20.9 and 20.5 cassettes contain this element in common. Results possibly indicate that gene expression during latency is a function both of elements within LATP2 together with those of other promoters such as LAP1 and MMLV.

In conclusion, virus 1764 pR20.9/UL43 would be suitable vector to use when relatively low level long-term transgene expression, at least up to 6months, would be required. This could be achieved either by footpad or sciatic nerve inoculation. Conversely, vector 1764 pR20.5/UL43 would be a suitable vector to use when high levels of transgene expression in the short term were required, particularly by sciatic nerve inoculation, but where long term expression is less important.
Figure 3.5. Gene delivery to peripheral ganglia following footpad inoculation with replication competent vectors. 3 vectors were tested in the study; i) 1764pR20.9/US5, ii) 1764pR20.9/UL43 and iii) 1764pR20.5/UL43 (see section 3.2.3. for details). 20μl of 1x10^8 pfu/ml of each virus was injected unilaterally into mouse rear footpad. DRG were removed at various times post inoculation, A. 3days B. 1week C. 2months and D. 6months, and examined for GFP expression under a fluorescent microscope or for β-galactosidase activity by X-Gal staining.
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A. 3 days

B. 1 week

C. 2 months

D. 6 months

i) 1764pR20.9/US5  ii) 1764pR20.9/UL43  iii) 1764pR20.5/UL43
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Figure 3.6. Gene delivery to peripheral ganglia following sciatic nerve inoculation with replication competent vectors. 2 vectors were tested in the study: i) 1764 pR20.9/UL43 and ii) 1764 pR20.5/UL43 (see section 3.2.3. for details). 2-5µl of 1x10^7 pfu/ml of each virus was injected unilaterally into the rear sciatic nerve of mice. DRG were removed at various times post inoculation, A. 3days B. 2weeks and C. 2months and examined for GFP expression under a fluorescent microscope and for β-galactosidase activity by X-Gal staining.
3.2.4. Deletion of Vhs.
The virion host shutoff protein (vhs) is a tegument protein and is responsible for the shutdown of host protein synthesis by the non-selective degradation of mRNA (Kwong et al. 1988). Vhs mutants have also been shown to prolong the half-life of viral mRNAs (Kwong et al. 1988; Oroskar and Read 1989). Vhs mutants have been shown to have a reduced pathogenicity in vivo but replicate normally in vitro (Strelow and Leib 1995). It was thus speculated that vectors deleted for vhs, concomitantly with ICP34.5 and VP16, might prove successful vectors for gene delivery to the PNS and thus should be tested. The deletion of multiple viral genes also provides an extra safety margin for safe and effective vectors. Previously in our laboratory, a virus deleted for ICP34.5, VP16 and vhs had been tested for gene delivery to lumbar ganglia following footpad inoculation, this work is now published (Palmer et al. 2000). Preliminary data had suggested that this virus might not be as efficient as one deleted for ICP34.5 and VP16 (1764), however further experiments were required.

3.2.4.1. Expression Cassettes and Vector Production.
Previously a 1764-based vector simultaneously deleted for vhs and UL43 had been created, termed 1764 UL43-MSVGFP vhs-pR15. Vhs was inactivated by the insertion of a reporter gene cassette, pR15 (Rob Coffin, UCL, London), at an NruI site (HSV nt 91854) and UL43 was inactivated by an MSV/GFP cassette at an NsiI site (HSV nt 94911). See figure 3.7. pR15 contains LAP1 (HSV nt 118181 to 118768), a ~700bp plasmid spacer sequence derived from pGem3Zf (Ndel-Xbal [Promega]), and an MMLVLTR promoter driving lacZ. The pR15 cassette was known to give latent transgene expression when inserted either into vhs or UL43 (here called pR8c) as shown previously (Palmer et al. 2000).
3.2.4.2. Deletion of Vhs Reduces the Efficiency of Reporter Gene Expression In Vivo Following Peripheral Administration.

The vector 1764 UL43-MSV/GFP vhs-pR15, was tested \textit{in vivo} for gene delivery to peripheral ganglia following footpad and sciatic nerve inoculation (see figure 3.8). Results show that reporter gene expression is only efficient during the acute stage of viral infection, at 3 days p.i. and at later time points reporter gene expression drops. Following sciatic nerve inoculation, and during latent infection, no GFP or \textit{lacZ} expression is observed and after footpad injection only low levels are observed, at 1 month p.i. which decline to undetectable levels at 5 months p.i. This result suggests that deletion of vhs does not enhance gene delivery to DRG following peripheral administration and is consistent with similar experiments performed earlier in the laboratory (Palmer \textit{et al.} 2000).

Since the pR15 cassette previously supported latent transgene expression (Palmer \textit{et al.} 2000), these results would suggest that the lack of GFP or \textit{lacZ} expression is here a result of failure of the virus to achieve a latent infection. This theory is substantiated by the fact that a vhs mutant was shown to have a reduced ability to establish latency in trigeminal ganglia compared to parental KOS virus (Strelow \textit{et al.} 1997). Deletion of vhs together with ICP34.5 and VP16 may therefore further reduce the establishment of latency compared to a vhs deletion alone. To confirm this theory it will be necessary to quantify the amount of virus establishing a latent infection. This could be done by performing ISH for LAT RNA, or RT PCR or ISRTPCR for HSV-DNA (see section 1.4.3.8.).

In conclusion these results suggest that deletion of vhs together with deletion of ICP34.5 and VP16 inactivation, is not advantageous in the vector described here. However this result may change if different expression cassettes, containing LATP2, were to be used in a similar vector backbone.
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A. 1764

Figure 3.7. Expression cassettes and replication competent vector backbone. A. 1764 vector backbone deficient in ICP34.5 and VP16 and showing insertion sites in UL43 and vhs. B. The pR15 cassette. C. An MSV/GFP expression cassette. In the same vector B. and C. were inserted into vhs and UL43 genes respectively and at the positions shown. The vector was tested for gene delivery to DRG following peripheral administration (see text for details). MSV, murine sarcoma virus promoter. MMLVLTR, Moloney murine leukemia virus promoter. GFP, Green fluorescent protein. LacZ, β-galactosidase. LAP1 is a HSV latency associated promoter. Arrows indicate orientation of the promoters.
Figure 3.8. Gene delivery to peripheral ganglia following footpad or sciatic nerve inoculation with a replication competent vector deleted for vhs. 20μl of 1x10⁶pfu/ml of virus was injected unilaterally into the rear sciatic nerve of mice. DRG were removed at various times post inoculation, A. 3days B. 2weeks Bii. 1week C. 2months and D. 5months and examined for GFP expression under a fluorescent microscope and for β-galactosidase activity by X-Gal staining.
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1764 UL43-MSVGFp vhs-pR15

Footpad

A. 3 days

Bi. 2 weeks

C. 2 months

D. 5 months

Sciatic Nerve

Bii 1 week
3.2.5. Estimation of the Efficiency Of Vector Uptake by Ganglionic Neurons that Project to the Footpad.

The level of reporter gene expression observed following footpad inoculation of vector was often variable between animals, however the highest levels observed remained consistent. To define the number of neurons projecting from the footpad to the lumbar ganglia, and thus determine if footpad inoculation of vector and ensuing vector uptake by nerve terminals was efficient, the axonal retrograde tracer Fluororuby was used (see section 3.1.2.). An aqueous solution containing various volumes of Fluororuby was injected into mice, 2 per different volume, via footpad inoculation. After 1 week, mice were sacrificed and Fluororuby-containing cells were determined.

3.2.5.1. In Vivo Analysis of Fluororuby Uptake.

Results in figure 3.9 show that at all the doses of Fluororoby used, there were no differences in the number of Fluororuby positive cells detected. This would indicate that a saturation point had been reached, even at the lowest dose, meaning that all possible available cells had taken up the tracer. The results provide us with an indication of the distribution of lumbar ganglion cells that project to the footpad and this should reflect the number of cells that it is possible to target following footpad inoculation of a vector.

In order that the results about Fluororuby uptake can be compared to the efficiency of vector uptake, this work will need to be repeated such that animals are injected with tracer alongside a control group that are injected with virus. However the study presented here was intended to be a quick look-see, in order to check that our results for vector uptake were within the correct expected range for the number of available neurons. At similar times post inoculation the number and distribution of lacZ positive cells seen following footpad injection of vectors (see figures 3.3 and 3.5) was similar to those containing Fluororuby.

Previously a similar result was obtained using the tracer Fluorogold (Ramakrishnan et al. 1996). Here the authors found in a rat model that the distribution of neurons containing Fluorogold was similar to those of the neurons containing HSV-1 DNA or LAT, as detected by RTPCR or ISRTPCR.
respectively. Thus the HSV vectors described in this study appear to be
effective in targeting all possible available cells. This is promising if they are to
be used to deliver therapeutic genes or to study gene function.
Figure 3.9. Ganglionic neurons and sciatic nerve after retrograde axonal labelling using Fluororuby. A-D. Mice were inoculated unilaterally in the rear footpad with increasing volumes of a 10% Fluororuby solution A. 2.5μl B. 5μl C. 7.5μl D. 10μl. 1 week post inoculation DRG and sciatic nerve were removed and observed under UV light at a wavelength of 590nm. The number of cells labelled with Fluororuby is similar in A through D and the distribution is comparable to that observed with reporter genes following viral vector inoculation in the footpad.
3.2.6. Strategies to Avoid Possible Immune Responses Following Vector Administration.

It was unclear whether the observed drop in transgene expression during latent virus infection was the result of viral clearance due an immune response (to either viral antigens and/or encoded reporter genes), to promoter shut-off or to vector toxicity. Thus a number of strategies were employed to investigate these possibilities.

3.2.6.1. Immunosuppression using Cyclosporin.

The efficiency by which a virus continues to express genes during latency may be affected by the immune response, such that immunosuppression could increase the number of neurons harbouring a latent infection. Cyclosporin is a drug that functions to block lymphocyte activation (Sigal and Dumont 1992). In these experiments it was hypothesised that cyclosporin treatment of mice might cause transient immunosuppression of animals and function at two levels; firstly by blocking any cellular or humoral immune response following peripheral administration and thus increasing latent infection; secondly, after latent infection has occurred, to block any immune response to virus and/or encoded transgenes.

3.2.6.2. Vector Choice for In Vivo Immunosuppression Analysis.

Since previous work highlighted two potential vectors (section 3.2.3) that gave efficient gene delivery following sciatic nerve and footpad inoculation, 1764pR20.5/UL43 and 1764 pR20.9/UL43 respectively, these vectors were used in this immunosuppression study. For each virus 6 mice were used. Animals were treated with cyclosporin from -2 to 28 days p.i. (every 2 days), as detailed in section 3.1.1. At day 0 virus was administered unilaterally via either the footpad or sciatic nerve inoculation route. 2-5μl of 1764 pR20.5/UL43 (1x10^6 pfu/ml) was injected in the sciatic nerve and 20μl of 1764 pR20.9/UL43 (1x10^6 pfu/ml) was injected in the footpad. At 3 days and 28 days p.i., 3 mice for each vector were sacrificed and DRG were removed and GFP and lacZ activity observed. Results can be seen in figure 3.10.
3.2.6.3. Cyclosporin Treatment does not Affect Reporter Gene Expression.

If reporter gene expression observed in figure 3.10 is compared with the corresponding reporter gene expression observed in figures 3.5 and 3.6 it can be seen that transgene expression declines in a similar manner between treated (immunosuppressed) and untreated animals. Although this comparison is made between different sets of experiments the frequency with which immune competent mice had previously been inoculated with vector, and our knowledge of the results, made this a viable comparison. In addition we were looking for a noticeable difference in gene expression and were not at this point concerned with slight differences that might be seen if quantification had been performed.

Results here would suggest that if the reduction in transgene expression is brought about by an immune response, this is not suppressed by cyclosporin. It is possible that optimisation of the cyclosporin dose and frequency of delivery may improve results. However, a similar result has been found previously when using cyclosporin as an immunosuppressant (Halford and Schaffer 2000). Here the authors used cyclosporinA (CsA) treatment in an effort to increase the establishment of latency in trigeminal ganglia (TG) of an ICP0 deleted virus. They administered CsA daily from -3 to 13 days p.i and measured replicating virus in the eye and viral load in trigeminal ganglia. They showed that CsA treatment consistently failed to enhance the levels of viral DNA in TG. However the same group also used an alkylating agent, cyclophosphamide (CyP), that causes transient lethal DNA damage and thus decreases the white blood cell (WBC) population. They showed that this treatment restored a WT latent phenotype on the ICP0 virus. CyP treatment is however a harsh regime to consider, since the high dose required (200mg/kg/day) was often lethal in this mouse model. Nevertheless it highlights that immunomodulation can increase the efficiency with which HSV vectors enter latency. Consistent with these findings, a group using cyclosporin treatment to modulate the immune response to adenoviral vectors, found that there was no quantitative difference in β-galactosidase staining after administration of Ad vectors to the hippocampus in immunosuppressed or vehicle treated animals (Geddes et al. 1996).
Previously it has been reported that cyclosporinA treatment reduces the inflammatory response to a multi-mutant HSV-1 vector (Mabon et al. 1999). Here a virus deleted for ICP4 and inactivated for VP16 and vhs (Johnson et al. 1994) was used. CsA treatment was shown to improve the persistence of lacZ activity in the hamster after injection into the adrenal gland and retrograde transport to adrenal sympathetic preganglionic neurons. Here 50mg/kg of CsA was delivered intramuscularly on a daily basis. This study provides evidence that CsA treatment can improve transgene expression from HSV-1 vectors. However it should be noted that in this study a different animal (hamster) and model were used, compared to the study described in this thesis.

The results presented here demonstrate that in this study transient immunosuppression of mice using cyclosporin did not affect reporter gene expression to any observable extent.
Figure 3.10. Effect of transient immunosuppression on the efficiency of reporter gene expression from HSV vectors in mice. A. 2-5 μl of 1764 pR20.5/UL43 (1x10^8 pfu/ml) was injected unilaterally in the rear sciatic nerve. B. 20 μl of 1764 pR20.9/UL43 (1x10^8 pfu/ml) was injected unilaterally in the rear footpad. Cyclosporin was administered (every two days) from -2 to 28 days p.i. DRG were removed at 2 days and 1 month post virus inoculation and GFP and lacZ expression determined.
3.2.6.4. Removal of lacZ from the pR20.9 Cassette.

It was hypothesised that the lacZ protein could be responsible for a cell-mediated immune response and thus a decline in latent reporter gene expression. It is known that the β-galactosidase protein is fairly stable and has a half-life of 43hrs in human fibroblasts (Ko et al. 1983), in addition lacZ is a large protein when compared to GFP. For these reasons the protein may elicit a strong immune response. To test this theory, the β-galactosidase gene in the pR20.9/UL43 cassette was replaced with blue fluorescent protein (BFP) with the aim of creating a virus containing both BFP and GFP, which might be less immunogenic than one containing GFP and lacZ.

3.2.6.5. Expression Cassette and Vector Production.

A NtotI/T4Polymerase/Nhel BFP fragment was excised from pQBI-50 (Qbiogene [Kramel Biotech. Northumberalnd, UK]) and cloned into pR20.9/UL43 between EcoR1 and Xbal replacing lacZ. The resulting plasmid contains BFP driven by the LAP1 promoter and GFP driven by the MMLV-LTR (see figure 3.11). A virus was produced by co-transfection of the pR20.9/UL43/BFP plasmid with 1764 viral DNA following protocol 2.2.4. Recombinant green plaques were purified from white plaques. During the purification procedure it became apparent that the virus could not be purified beyond approximately 80% green plaques. Sequence alignment showed that BFP gene had a 74.93% DNA nucleotide identity to EGFP (see figure 3.12), which at the amino acid level increased to 97.49%. Thus it is probable that during purification, recombination events occurred between BFP and GFP sequences introducing mutations into the genes and thus rendering both proteins non-functional. This result was not followed up by replacement of lacZ with other genes such as chloramphenicol acetyltransferase (CAT) to test if lacZ did indeed contribute to immunogenicity. However the ease with which BFP expression can be detected, like GFP and lacZ, made the gene an appropriate candidate to try. It remains to be seen to what extent the lacZ protein, and indeed other reporter genes, contribute to any immune response.
Figure 3.11. Schematic diagram to show the 1764 vector backbone and pR20.9 expression cassette containing blue fluorescent protein. A. 1764 vector backbone showing insertion site in UL43. B. pR20.9 expression cassette. The cassette contains BFP in place of lacZ. See section 3.6.2. for details. MMLVLTR, Moloney murine leukemia virus long terminal repeat. GFP, green fluorescent protein. LAP1 and LATP2, latency associated promoters. Arrows indicate the orientation of the promoters.
Figure 3.12. Nucleotide sequence homology between EGFP and BFP. Adapted from www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html.

E/GFP accession number U76561. BFP sequence taken from pQBI50f (www.qbiogene.com) The two sequences show a 74.930% nucleotide homology which increases to 97.49% similarity at the amino acid level.
3.2.7. Detection of Latent HSV-1 in Lumbar Ganglia by In Situ Hybridisation.

Since results were inconclusive with regard to the immune response and its influence on gene expression, it was decided to test to what extent promoter inactivation may be responsible for the decline in latent transgene expression. As discussed earlier latent HSV can be quantified by detection of the latency associated transcripts or by direct detection of latent vector genomes. Previously other groups have used in situ hybridisation (ISH) to detect latent viral genomes, using either a DNA probe (Dobson et al. 1989; Carpenter and Stevens 1996; Lokensgard et al. 1994) or a riboprobe (Ecob-Prince et al. 1995; Lachmann and Efstathiou 1997a). Lokensgard et al performed ISH using probes for LATs and lacZ mRNA and found that whilst 3 different viruses established a latent infection with equal efficiency, only one of these vectors (containing a LAP1/MMLVLTR fusion cassette in the gC locus) was positive for LacZ mRNA. Similarly Ecob-Prince et al found that 1 of 4 viruses tested, that containing the CMV promoter driving lacZ, was positive for β-galactosidase mRNA during latency whilst all 4 were positive for LATs and thus a latent infection. Based on these findings we decided to use ISH techniques (using a DNA probe complementary to the LAT region, [section 3.1.3.4]) in order to detect latent HSV in lumbar ganglia and see if results correlated with lacZ activity measured by X-Gal staining.

3.2.7.1. Vector Choice for Detection of Latent HSV.

Since previous work highlighted two vectors that gave efficient gene delivery after footpad and sciatic nerve inoculation, these vectors were chosen for this study, 1764pR20.9/UL43 and 1764pR20.5/UL43 respectively. Of particular interest was the pR20.5 virus, since this virus gave high levels of transgene expression during acute infection that dropped considerably during latent infection. In addition to these vectors, two vectors acting as controls were also studied, which did not express any transgene; a) 1764 and b) 1764LATP2NSElacZ/UL43 (see figure 3.13.). Thus levels of LAT could be observed without any possible toxic effects of lacZ and/or GFP expression. The 1764LATP2NSElacZ/UL43 virus provided a control for inactivation of the UL43 gene. For undefined reasons the vector containing a LATP2NSElacZ cassette
in UL43 had previously been shown in the laboratory to give no lacZ expression in vivo (Mike Robinson, personal communication). Vectors were injected into mice; 20µl each of 1x10⁶ pfu/ml of 1764, 1764LATP2NSE/acZ/UL43 and 1764 pR20.9 into the footpad and 2-5µl of 1x10⁷ pfu/ml of the pR20.5 vector into the sciatic nerve. DRG from 3 mice per vector were removed at 3 days and 1 month post inoculation and either X-Gal stained for lacZ activity or sectioned and processed via ISH for LAT detection (see section 3.1.3.).
Figure 3.13. Schematic diagram to show vector controls used for in situ hybridisation experiments. A. 1764 vector deleted for both copies of ICP34.5 and deficient in VP16. B. 1764 P2/NSE/lacZ/UL43, a 1764 vector with a reporter cassette inserted at the UL43 locus. LATP2, HSV-1 latency associated promoter. NSE, Neuronal specific enolase promoter. LacZ, β-galactosidase. PA polyadenylation sequence.
3.2.7.2. Decline in Transgene Expression During Latent Infection does not Appear to be a Function of Promoter Shutdown.

The results of *in situ* hybridisation can be seen in figures 3.14A and 3.14B. Figure 3.14A shows the results for the control viruses, 1764 and 1764LATP2NSElacZ/UL43, which do not express reporter genes. In both cases the viruses show similar numbers of LAT positive cells at 3days and 1 month p.i. This is an interesting observation and suggests that the viruses were able to establish a latent infection at levels similar to that observed during acute infection, and that viral clearance is not observed. In addition, the signal from LAT positive cells is stronger at 1 month p.i. Since the sections were processed in parallel and were left to hybridise to the LAT probe for exactly the same amount of time, this observation is not an artefact of differential hybridisation time. This suggests, as expected, that during latency LAT production increases compared to that seen during lytic infection.

The photographs seen in figure 3.14B show results from vectors 1764pR20.9/UL43 and 1764pR20.5/UL43. Since each of these viruses expresses *lacZ*, DRG were extracted at 3days p.i. and whole-mount X-Gal stained for *lacZ* activity, as a measure of acute expression. At 1 month p.i. DRG were extracted and either X-Gal stained for *lacZ* activity or processed for LAT detection via ISH (see section 3.1.3.). X-Gal staining shows that reporter gene activity decreases from both viruses between 3 days and 1 month p.i., although to a lesser extent with the pR20.9 cassette, and confirms results described earlier (see section 3.2.3.4.). *In situ* hybridisation to detect LATs suggest that for the pR20.9 virus the number of LAT-positive cells and *lacZ*-positive cells are similar, thus indicating that the promoters in the pR20.9 cassette are efficient at maintaining gene expression during latent infection and comparable to the efficiency of LAT expression itself. However, the results from the pR20.5 vector indicate that there are more LAT-positive cells than *lacZ*-positive cells, indicating that the promoters in this cassette are less efficient at maintaining latent transgene expression. It is also notable that with both transgene expressing vectors, the numbers of LAT positive cells at 1 month are considerably lower than with the two control viruses. This suggests that cells harbouring virus genomes expressing *lacZ*/GFP are more affected by the host
immune system or other effects than are non-marker gene expressing viruses, even though the use of cyclosporin did not appear to have an effect. It would have been interesting to perform ISH for LATs at 3days p.i. to see how these correlate with lacZ activity at the same time point.

One drawback of these results is that at 1 month p.i. consecutive sections were not stained for LAT expression and lacZ activity. This would have provided a more accurate comparison between lacZ and LAT expressing cells rather than having to rely on whole mount X-Gal staining. In addition quantification of results (of both lacZ and LAT positive cells), would have provided a more accurate measure of promoter inactivation.

It has been suggested previously that within mouse trigeminal ganglia detection of LATs by ISH does not necessarily correlate with the detection of latent viral DNA (Mehta et al. 1995). Here it was found that many more neurons harboured latent viral genomes than could be detected by ISH for LATs. It has since been found that different strategies to detect LATs such as contextual analysis, CXA (Sawtell 1997) or in situ reverse transcription polymerase chain reaction, ISRTPCR (Ramakrishnan et al. 1996)(see previous), increase the sensitivity of LAT detection and thus increase the correlation between LAT-positive and genome-positive neurons. The relevance of this information to this thesis is that although in situ hybridisation may not readily detect all latent viral genomes, those detectable by this means should correlate with those exhibiting detectable lacZ activity. If the level of LAT detection is dependent upon the number of copies of the latent genome or an active transactivator or repressor then it might be expected that LAT expression and lacZ activity might be a function of the same effect.
Figure 3.14A. *In situ* hybridisation to detect LAT RNA in lumbar ganglia. Mice were infected via footpad inoculation with vectors: i) 1764 and ii) 1764 LATP2NSE/lacZ/UL43 (see section 3.7.2. for details) and their DRG removed, sectioned and fixed. A-B DRG extracted at 3days p.i. and 1month p.i. respectively and probed with a LAT specific DNA probe. C. Non-specific labelling with the LAT probe.
Figure 3.14B. *In situ* hybridisation to detect LAT RNA in lumbar ganglia. Mice were infected via footpad inoculation with vectors; i) 1764 pR20.9/UL43 and ii) 1764 pR20.5/UL43 (see section 3.7.1. for details). A-B DRG extracted at 3days p.i. and 1month p.i. respectively and X-Gal stained for lacZ activity C. DRG extracted 1month p.i and probed with a LAT specific DNA probe. D. Non-specific labelling with the LAT probe.
3.3. DISCUSSION.
There have been many reports describing gene delivery to peripheral ganglia using HSV-1 vectors (see introduction). However, many of these reports use vectors deleted for gC and tk and whilst these vectors are attenuated compared to wild type they are still pathogenic. The initial aim of this study was to further identify parameters and possible gene deletions that allow gene delivery to peripheral ganglia.

In support of previous findings (Coffin et al. 1996) the results show that a vector with a mutation in VP16 transactivation and deletions in both copies of ICP34.5 (1764), is a suitable vector to allow gene delivery to peripheral ganglia, following both footpad and sciatic nerve inoculation. This vector was able to efficiently establish a latent infection as seen by in situ hybridisation for LATs and thus support latent transgene expression as detected by X-Gal staining for lacZ activity. It was further established that deletion of vhs did not increase the efficiency of transgene expression, either during lytic or latent infection. This however may be a reflection of the expression cassettes used rather than the vector backbone.

1764 is a safe backbone to use, as in cell culture it cannot revert to the wild-type phenotype since the mutated genes, ICP34.5 and VP16, are not required to be supplied in trans. In addition the deletions in both of these genes means that the vector is of greatly reduced pathogenicity. ICP34.5 is a virulence factor (Chou et al. 1990; Valyi-Nagy et al. 1994) and VP16 is responsible for the transactivation of IE genes (Campbell et al. 1984). However the usefulness of the replication competent 1764 backbone in gene therapy protocols is likely to be limited because the virus can still replicate in dividing cells. It might be expected that for most clinical gene therapy protocols HSV vectors would have multiple mutations in essential genes. Thus if patients were already carrying latent HSV, recombination would not produce a wild-type virus carrying therapeutic genes, either by repair of the delivered vector or mutation of the wild-type virus. Nevertheless the 1764 backbone will prove useful in terms of research. Little is known about the ability of replication incompetent vectors to
reach ganglia following peripheral administration. Deletion of essential genes in vectors for the PNS will be discussed in chapter 4.

A second aim of this study was to identify the optimal vectors and promoter combinations that allow latent transgene expression. Collectively the results show that the LAT region is capable of allowing long-term gene expression in a relatively non-promoter-specific fashion. Thus the LATP2 region or elements therein, was capable of conferring latent transgene expression, at least up to 6 months in some cases, on the MMLVLTR, the CMV promoter and the LAP1 promoter itself (and previously in our laboratory a minimal NSE promoter, but not the full length NSE promoter [Mike Robinson, Personal communication]). This was evident both in the endogenous LAT region and at ectopic sites in the genome (UL43 and US5). However as observed using the pR20.5 cassette, elements as well as those located in LATP2 are involved in latent gene expression, perhaps in LAP1. In support of this data previous work has shown that LAP2 can confer a long term enhancer effect on LAP1 (Lokensgard et al. 1997), whereas LAP2 gives weak latent transgene expression alone (Goins et al. 1994). In addition MMLV LTR appears to be able to confer long-term activity to the LAP1 promoter (Lokensgard et al. 1994).

Currently in our laboratory work is underway to determine which parts of the 1.4kb LATP2 region are responsible for the long-term enhancer effects observed. In addition to this we have found that elements located in LAP1 between bases -343 and -603 (with respect to the transcriptional start site [HSV nt 118180 to 118440]) are necessary for the long term enhancer capabilities of LAP1 on the MMLV promoter (Palmer et al. 2000).

As seen with the pR20.9 cassette the putative long-term enhancer elements of LATP2 function in a bidirectional manner supporting both lacZ and GFP expression. This observation is supported by others (Berthomme et al. 2000; Lokensgard et al. 1997). Collectively the foregoing evidence suggests that the LAT region might function to remodel the surrounding chromatin and thus neighbouring promoters and function in this manner to allow latent transgene expression.
It is known that the β-galactosidase protein remains detectable for some time after production, due to its high stability (Margolis et al. 1993a). It has previously been suggested that histochemical detection of β-Gal enzymatic activity at early time points during latent infection (21 days) is an unreliable measure of promoter activity (Margolis et al. 1993b). Here it was found that whilst histochemical detection of β-galactosidase activity was apparent at 21 days p.i., no lacZ transcripts could be detected. Thus in this case histochemical analysis of enzymatic activity was misleading. The same group suggested that histochemical analysis at 42 d.p.i. would allow for turnover of the β-galactosidase protein and thus detection was not due to perisitance of the enzyme (Lokensgard et al. 1994). In this thesis lacZ activity was determined histochemically during latency, at time points of 1, 2, 5 and 6 months p.i. Since time points of 2 months were used in most cases, it would seem reasonable that results obtained were that of authentic lacZ transcription. In addition it would seem unlikely that the protein could persist for periods up to 5 or 6 months. This does not preclude the fact that at time points of 1 month or earlier, histochemical staining could be a function of slow protein turnover. However since results at 1 month generally matched and were consistent with those at later time points, this would suggest that at 1 month results were authentic also.

All of the vectors described in this chapter showed, to varying extents, a decline of transgene expression over time, consistent with the establishment of latency. The reasons for this drop in expression were ambiguous and thus several attempts were made to try understand them, for example immunosuppression of animals, changing the transgene and determining if the drop in transgene expression was comparable to a drop in the establishment of latency. Results obtained from ISH experiments indicated that both promoter shutdown and a reduction in the number of cells harbouring a latent infection were responsible for the drop in transgene expression observed. At present the affects of the immune response and viral infection and establishment of latency remain unclear, as cyclosporin treatment of animals did not appear to improve results. Logically the next set of experiments should focus on further disabled viruses and thus vectors that are replication incompetent, in order to establish if such
less toxic vectors will improve results. This will indicate if the immune response is involved in the drop of transgene expression. See chapter 4.

The ability to express two transgenes from a single locus, as seen with the pR20.9 and pR20.5 cassettes, is an attractive feature of the vectors described here. The expression of both GFP and lacZ, as seen from the pR20.9 cassette, provides the first evidence of two heterologous genes being expressed during HSV latency. Together with the ability of HSV to incorporate large inserts this provides an attractive vector system for future development. Furthermore, HSV is ideally suited for gene delivery to the PNS since it has evolved the capability of retrograde transport to ganglia following peripheral infection, a feature not observed with other viral vectors. In the PNS HSV could, for example, be used as a vector to treat ALS, nerve regeneration, various pain states and to study the processes and factors involved in any of these conditions, each requiring nerve trafficking to have occurred.
CHAPTER 4:

IDENTIFICATION OF OPTIMAL REPLICATION INCOMPETENT VECTORS FOR GENE DELIVERY TO THE PNS
CHAPTER 4:
IDENTIFICATION OF OPTIMAL REPLICATION INCOMPETENT VECTORS FOR GENE DELIVERY TO THE PNS.

4.0. INTRODUCTION.
Previously there has been little reported work documenting the use of replication incompetent HSV vectors and gene delivery to the peripheral nervous system. The majority of reported vectors used have been those deleted for gC or tk and whilst these vectors are attenuated for growth compared to wild type virus, they are still cytotoxic in most cell types. The first reported replication incompetent vector used in the PNS was described by Dobson et al. (Dobson et al. 1990), who demonstrated that a vector with an MMLVLTR/lacZ cassette inserted into ICP4, was able to establish both acute and latent infections in sensory ganglia (L4 and L5). LacZ positive neurons were detected up to 24 weeks, the longest time point studied. They reported that β-galactosidase labelled neurons, as determined by histochemical staining, were more abundant after sciatic nerve inoculation rather than footpad inoculation, although only data representing sciatic nerve inoculation was shown. However, the number of lacZ positive cells in these experiments at any time point was very low. As mentioned in chapter 3, it was speculated that virus replication would be required in order to achieve a latent virus infection in peripheral ganglia. Results such as those presented by Dobson et al would support this hypothesis, as only very low gene transfer was seen. Here, the need to perform sciatic nerve injection in order to achieve reasonable gene delivery to DRG suggests that peripheral replication is required for a vector to reach nerve terminals following footpad inoculation.

More recently a conditionally replication incompetent vector capable of gene delivery to the PNS has been reported (Marshall et al. 2000). Here the virus was inactivated for VP16, as described previously (Ace et al. 1989), contained a partial deletion in ICP0 (which does not prevent replication) and a temperature sensitive (ts) mutation in ICP4. The mutation in ICP4 allowed replication at the permissive temperature of 31°C, but reduced replication significantly (10\(^5\)-fold) at the fully non-permissive temperature of 38°C. The vector contained an IRES
β-geo (a fusion between the β-galactosidase gene and the neomycin resistant gene) cassette inserted into the LAT region. The group found that following footpad inoculation of vector, histochemical detection of β-galactosidase in the DRG was evident at 3 days and increased through 25 days, remaining consistent at 5 months. This study concluded that replication was probably not required for the establishment of a latent infection in DRG following footpad inoculation, since gene delivery was achieved even without detection of replicating vector. This therefore conflicted with the previous hypothesis that replication may be necessary for virus to reach DRG following peripheral administration. However, as the mouse footpad is likely to be below the fully non-permissive temperature of the virus, 38°C, replication was possible even though it was not detected.

There have been reports of other non-replicating HSV vectors which have been deleted for various combinations of IE genes (Wu et al. 1996; Samaniego et al. 1997; Krisky et al. 1998; Samaniego et al. 1998). Collectively these vectors have demonstrated that deletion of multiple IE genes is necessary to minimise vector toxicity in vitro and thus it was reasoned that following further development such replication incompetent vectors would be of use in gene therapy protocols. However, as yet none of these vectors have been reported as having been tested in vivo for gene delivery to the PNS and so their use in this capacity remains to be seen. As a preliminary investigation Krisky et al performed in vitro studies using primary DRG cultures. They found that following infection with a triple mutant for ICP4, ICP22 and ICP27, cells survived for 21 days and transgene expression persisted in some cells for at least 14 days, indicating the potential of this type of vector in these circumstances.

In this chapter, studies are performed with replication incompetent vectors, testing their ability to reach spinal ganglia following peripheral administration. Since viruses impaired for IE gene expression are known to have reduced toxicity (Samaniego et al. 1998), it might be appropriate to use such vectors for peripheral gene delivery, if they were found to be effective, as they would probably have an increased safety profile. In addition, the multiple deletion of essential genes means that if such vectors are to be used in clinical situations then any recombination between replication incompetent vectors and wild type
virus should only result in the generation of further replication incompetent virus. Importantly, no wild type virus carrying the therapeutic gene should be produced.
4.1. RESULTS.

4.1.1. Vectors with a Deletion in the Essential Gene ICP27.
ICP27 is an essential IE gene (Sacks et al. 1985) encoding a phosphoprotein. ICP27 mutants have been shown to display a variety of phenotypes as a result of the numerous regulatory functions the protein performs. Mutants do not replicate owing to a profound decrease in viral DNA synthesis and late gene expression (McMahan and Schaffer 1990; Sacks et al. 1985). In addition there is an over expression of some immediate early and early genes (McCarthy et al. 1989; Rice and Knipe 1990; McMahan and Schaffer 1990; Smith et al. 1992).

ICP27 mutants also show an impairment of host protein shut-off since ICP27 normally acts to inhibit pre-mRNA splicing (Hardy and Sandri-Goldin 1994; Hardwicke and Sandri-Goldin 1994). The ICP27 protein is also toxic in vitro (Johnson et al. 1994). This information suggests that ICP27 deleted vectors are appropriate for testing in this chapter in that they require complementation in trans in order to replicate and thus will be replication incompetent in vivo. In addition, infected cells would not be subjected to any toxic effects of ICP27 expression, including the inhibition of pre-mRNA splicing.

Previous work in our laboratory has shown that a virus deleted for ICP34.5 and ICP27 can infect CNS neurons of rodents and primates in vivo with high efficiency (Howard et al. 1998). Here it was observed that a vector inactivated for ICP27 and ICP34.5 gave higher gene delivery efficiency than the ICP34.5 single mutant and in addition produced less damage around the site of injection in vivo.

4.1.1.1. Vector Production.
Two vectors had previously been constructed; 1764 P2-27-/vhs-pR20.9 and 1764 P2-27-/vhs-pR20.5. Both vectors were based on the 1764 backbone virus deficient for ICP34.5/OrfP and VP 16, as described earlier. In addition both vectors were deleted for the endogenous LATP2 region (HSV nt 118768 to 120470 Ddel to Hpal) (Lilley et al. 2001) and the ICP27 gene (HSV nt 113272 to 116869, MluI to MluI [this also deleted the nonessential genes UL55 and UL56]) (Howard et al. 1998). Each vector was insertionally inactivated for vhs by
insertion of an expression cassette, either pR20.9 or pR20.5 (see section 3.2.3.2.), at a unique \textit{NruI} site in the \textit{vhs} gene (HSV nt 91854). See figure 4.1.

\textbf{4.1.1.2. In Vitro Vector Analysis on Permissive Cells.}

Vectors were propagated on B130/2 cells (Howard \textit{et al.} 1998). B130/2 cells are a BHK-derived cell line complementing the ICP27 deletion. VP16 was complemented by addition of 3mM HMBA to the media. During lytic replication on B130/2 cells the pR20.5 cassette expressed GFP and \textit{lacZ} at high levels, as observed when the same cassette was inserted into UL43 flanking regions in a 1764 backbone (see figure 3.3.). However, the phenotype of the pR20.9 cassette was different to that previously described as both GFP and \textit{lacZ} expression were strong. When the pR20.9 cassette was inserted into UL43 or US5 flanking regions in a 1764 backbone, only low levels of \(\beta\)-galactosidase activity were detectable upon X-Gal staining (see section 3.2.3.3.). Furthermore it has been found in our laboratory (Caroline Lilley, unpublished observations) that when the pR20.9 cassette is inserted into \textit{vhs} flanking regions, in a vector also deleted for ICP4, ICP27 and LATP2, \textit{in vitro} the cassette once again expresses \textit{lacZ} and GFP at high levels. At present the reasons for the inconsistent phenotype of pR20.9 is unknown. It is possible that repression of LAP1 mediated by ICP0 or ICP4 and sequences within LATP2, as suggested previously, does not occur when the cassette is inserted into the \textit{vhs} gene. Or, when inserted into a replication incompetent vector, insufficient levels of ICP4 and/or ICP0 are produced to mediate the repressive effects previously observed in 1764.
Figure 4.1. Expression cassettes and replication incompetent vector backbone. A. 1764 P2/-27/-vhs- vector backbone deficient in LATP2 ICP27, vhs, ICP34.5 and VP16 (see section 4.1.1.1. for details) B. pR20.9 in vhs flanking regions. C. pR20.5 in vhs flanking regions. RSV, Rous sarcoma virus promoter. MMLVLTR, Moloney murine leukemia virus promoter. GFP, Green fluorescent protein. LacZ, β-galactosidase. CMV, cytomegalovirus promoter. LAP1 and LATP2, HSV latency associated promoters. Arrows indicate direction of promoters.
4.1.1.3. In Vivo Gene Delivery with a Vector Deleted for ICP27.

The two vectors 1764 P2-/27-/vhs-pR20.9 and 1764 P2-/27-/vhs-pR20.5 were tested for gene delivery to lumbar ganglia following peripheral administration. 2-5 μl of 1x10^7 pfu/ml of each vector were injected into mouse sciatic nerve and DRG extracted from 3 mice at 3 days, 1 week and 2 months post inoculation, (see figure 4.2). In addition 20 μl of 1x10^8 pfu/ml of the vector 1764 P2-/27-/vhs-pR20.5 was injected into mouse footpad and DRG extracted from 3 mice at 1 week post inoculation (see figure 4.3). Further time points for footpad inoculations were not performed as it was not anticipated from the previous results that gene expression would occur. Footpad inoculation of the pR20.9 vector was not performed. Sciatic nerve and footpad inoculations were performed using virus of the same titre as the replication competent vectors used in chapter 3, 1x10^7 pfu/ml and 1x10^8 pfu/ml respectively.

Results in figure 4.2 and 4.3 show that following sciatic nerve or footpad inoculation of either vector, gene delivery to peripheral ganglia is very poor. At all of the time points tested very few GFP or X-Gal positive cells could be detected. These results indicate that deletion of ICP27, and thus the use of a replication incompetent vector decreases the efficiency of gene delivery to peripheral ganglia to a significant extent. This would suggest that some level of replication competency is required in order for vectors to reach DRG either after sciatic nerve or footpad inoculation. Replication might be required for vectors to penetrate the various cell types in the footpad or those surrounding the axonal bundle, in order to reach the neuron. The finding that even after sciatic nerve inoculation vectors did not reach DRG, at the titre used here, was somewhat unexpected and contradicted results of Marshall et al. (Marshall et al. 2000) who suggested that replication is not necessary in order for vectors to reach peripheral ganglia. Results presented in section 4.1.2.2 would suggest that if higher titres of these viruses had been used for sciatic nerve inoculation, efficient gene delivery may have been observed although this was not tested with these viruses.
Figure 4.2. Gene delivery to peripheral ganglia following sciatic nerve inoculation of replication incompetent vectors deleted for the IE gene ICP27. 2 vectors were tested in the study; i) 1764 P2-27-/vhs-pR20.9. ii) 1764 P2/27-/vhs-pR20.5 (see section 4.1.1.3. for details). 2-5μl of 1x10^7 pfu/ml of virus were injected unilaterally into the rear sciatic nerve of mice. DRG were removed at various times post inoculation; A. 3days B. 1week C. 2months and examined for GFP expression under a fluorescent microscope and lacZ activity by X-Gal staining.
Chapter 4  
Replcation Incompetent Vectors

1764 P2- /27-

Vhs-/pR20.9  
Vhs-/pR20.5

A.  
3 days

B.  
1 week

C.  
2 months
Figure 4.3. Gene delivery to peripheral ganglia following footpad inoculation with a replication incompetent vector deleted for ICP27. 20\textmu l of 1x10^6 pfu/ml of vector 1764 P2-/27-/vhs-pR20.5 (see section 4.1.1.3 for details) was injected unilaterally into the rear footpad of mice and DRG were extracted after 1 week. GFP expression was examined under a fluorescent microscope and lacZ activity after X-Gal staining.

ICP4 is an essential IE protein that is absolutely required for viral replication (DeLuca et al. 1985). ICP4 functions to repress IE gene expression (Roberts et al. 1988) and to positively regulate the expression of E and L genes (DeLuca et al. 1985). Infection of non-permissive cells with ICP4 deletion mutants results in the expression of the four remaining IE genes, ICP0, ICP22, ICP27 and ICP47 plus ICP6 and OrfP, but no other HSV genes (DeLuca et al. 1985; Yeh and Schaffer 1993). It is known that ICP4 deletion mutants are toxic to cultured cells (Johnson et al. 1992) and this toxicity is largely caused by the IE proteins ICP0, ICP22 and ICP27 (reviewed in Lilley et al. 2001). An ideal vector would thus be deleted for ICP4 in combination with the remaining toxic IE genes. Such a vector was constructed and has been shown to be non-toxic to the cultured cells tested so far (Samaniego et al. 1998). Previously in our laboratory a vector deficient for ICP4, ICP27, VP16, and ICP34.5/OrfP had been constructed (Lilley et al. 2001) and has been shown to express minimal levels of IE genes in non-complementing cells and is thus also non-toxic. This vector is fully replication incompetent since it cannot replicate unless ICP4 and ICP27 are provided in trans. This vector was shown to give long-term gene delivery to CNS neurons in vitro and in vivo (Lilley et al. 2001) and was thus here tested for gene delivery capabilities in the PNS.

4.1.2.1. Vector Production.

Vector 1764 /27/-4- had previously been constructed in the laboratory. The vector was based on the 1764 backbone deleted for ICP34.5/OrfP and inactivated for VP16, as described earlier. In addition the vector was deleted for the ICP27 gene (HSV nt 113272-116869, MluI to MluI [this also deleted the nonessential genes UL55 and UL56]) and deleted in both copies of the ICP4 gene (HSV nt 126774 to 131730 [IRS] Sau3AI- Sphi) (Lilley et al. 2001). The vector had a pR19CMVlacZ cassette inserted into the LAT region. The cassette was cloned into the HSV-1 LAT region using a 3.5-kb NolI fragment (HSV-1 nt 118443 to 122029) inserted into pGem5 (Promega), the cassette cloned between two BstXI sites (HSV-1 nt 120219 to 120413). See figure 4.4. The vector was propagated on 27/12/M:4 cells, a BHK-derived cell line
complementing ICP4, ICP27 and VP16 (see section 2.2.2.3.) (Thomas et al. 1999b).

4.1.2.2. Reporter Gene Expression In Vivo using a Multiple IE Gene Deficient Replication Incompetent HSV Vector is a Function of Vector Titre and Delivery Route.

Figure 4.5. shows gene delivery to peripheral ganglia following inoculation with 1764 27-/I4- pR19CMVlacZ. The vector was injected via footpad and sciatic nerve inoculation routes at the same titres used previously for replication competent vectors, 2-5μl of 1x10⁸pfu/ml and 20μl of 1x10⁷pfu/ml respectively. DRG from 3 mice were extracted 1week post inoculation and examined for β-galactosidase activity by X-Gal staining. Results were as expected (see figure 4.5) and revealed that, like the vectors 1764 P2-/27-/vhs-pR20.9 and 1764 P2-/27-/vhs- pR20.5 (see 4.1.1.3.), very few X-Gal positive cells were apparent.

Following these somewhat disappointing results, it was hypothesised that by using the vector at a higher titre it might improve the gene delivery profile. Here the vector was used at a titre of 5x10⁸pfu/ml and inoculated either in the footpad or sciatic nerve, as before. DRG from 3 mice were extracted at 1week post inoculation and X-Gal stained to determine lacZ activity. Results can be seen in figure 4.5. It is apparent that using the vector at a higher titre significantly improved gene delivery to peripheral ganglia, most clearly following sciatic nerve inoculation. Thus, in conclusion, a fully replication incompetent vector can give efficient gene delivery to DRG, but only when used at a suitable titre and following sciatic nerve inoculation. Only low efficiency gene delivery to DRG is achieved via the footpad inoculation route, whatever the titre used.
Figure 4.4. Expression cassette and replication incompetent vector backbone. A. 1764 27/-4- vector backbone deficient in ICP27, ICP4, ICP34.5/OrfP and VP16 and showing insertion sites in the LAT regions. See text for details. B. pR19CMVlacZ expression cassette. The cassette was cloned into the HSV LAT region in a 3.5kb NotI fragment cloned into pGem5 (HSV-1 nt 118441 to 12202). Arrows indicate direction of the promoters.
Figure 4.5. Gene delivery to peripheral ganglia using a replication incompetent vector deleted for ICP4 and ICP27. Vector 1764 27/-4- pR19CMVlacZ was used in this study. A. and C. 20μl of virus was injected unilaterally into the rear footpad of mice at low titre of 1x10⁸ pfu/ml and high titre of 5x10⁸ pfu/ml. B. and D. 2-5μl of virus was injected unilaterally into mouse sciatic nerve at low titre of 1x10⁷ pfu/ml and high titre of 5x10⁸ pfu/ml. DRG were extracted 1 week post inoculation and DRG examined for lacZ activity following X-Gal staining.
4.1.2.3. Gene Expression from 1764 27/-4- pRI9lacZ is Maintained Over Time.

Following the observation that a high titre stock of vector 1764 27/-4- pR19CMVlacZ could efficiently reach peripheral ganglia following sciatic nerve inoculation (see section 4.1.2.2.), the virus was tested for long-term gene delivery to DRG. Here 2-5µl of a 1x10^8pfu/ml stock of vector was administered unilaterally into mouse sciatic nerve. DRG were removed from 3 mice at 2 days, 1 week and 1 month post inoculation and examined for lacZ activity following X-Gal staining. Results can be seen in figure 4.6 and show that transgene expression is maintained for at least 1 month and at levels equivalent to those seen at 2 days p.i. This result highlights, for the first time in this thesis, a vector that is able to successfully reach peripheral ganglia and maintain high levels of transgene expression during latent infection, without an apparent drop-off in expression levels.

Results in figure 4.6 also show that X-Gal staining produced a punctate speckled pattern in some neurons. This phenomenon was not observed in results presented elsewhere in this thesis, but has been reported previously (Dobson et al. 1990) (Ho and Mocarski 1989). Ho et al. suggested that this was because the β-galactosidase protein used contained no nuclear localisation signal and Dobson et al. suggested that β-Gal, the product of the histochemical reaction, was limited to membrane bound organelles in these neurons. However, the actual reason for such a speckled appearance remains to be determined.
Figure 4.6. Gene delivery to peripheral ganglia following sciatic nerve inoculation with a replication incompetent vector deleted for IC27 and ICP4. 2-5\mu l of 1x10^5 pfu/ml of vector 1764 27-4- pR19CMVlacZ was administered unilaterally to the rear sciatic nerve of mice. DRG were extracted at: A. 2 days, B. 1 week and C. 1 month post-inoculation and examined for lacZ activity following X-Gal staining.
4.2. DISCUSSION.

Previously there has been only one documented report of a vector deleted for an essential gene that has been used for gene delivery to peripheral ganglia (Dobson et al. 1990). Dobson et al. used a vector deleted for ICP4 and showed that lacZ expression was maintained for at least 24 weeks following sciatic nerve inoculation of virus. However here the efficiency of gene delivery was very low (an average of 60 cells per animal). In another report Marshall et al. (Marshall et al. 2000) used a conditionally replication competent vector that was inactivated for VP16 and ICP0 and had a ts ICP4 mutation. This gave transgene delivery following peripheral administration of the vector that increased during latent infection. Marshall et al. concluded that replication was therefore not necessary for HSV to reach DRG, since they expected their vector to be replication incompetent under the conditions used. However, since the mouse footpad is likely to be at a temperature somewhat below the fully non-permissive temperature for the ICP4 mutation (38°C), it is possible that the vector retained a degree of replication competence, which allowed gene delivery to occur. This possibility was however discounted by the authors since no replicating vector could be recovered from DRG of inoculated animals and in addition homogenised footpads from inoculated animals showed a drop in viral titre, consistent with a non-replicating vector. Based on the results presented in this thesis, using a number of replication incompetent vectors in the footpad, it would seem likely that replication was occurring in the vector described by Marshall et al.

Results presented here show that a fully replication competent vector deficient in ICP4, ICP27, VP16 and ICP34.5 when used at high titre and following sciatic nerve inoculation can efficiently reach DRG and express a delivered transgene for at least 1 month. The vectors 1764 27-/vhs-pR20.9 and 1764 27-/vhs-pR20.5 gave no gene delivery to DRG following sciatic nerve inoculation. However with hindsight improved results may be obtained if these vectors were also used at higher titres. Footpad inoculation of vectors deleted for the essential genes ICP27 alone or ICP27 and ICP4 gave very few lacZ positive neurons when used at a titre sufficient for delivery of replication competent vectors (1x10⁸ pfu/ml). An increase of titre did not result in efficient gene delivery. These
results collectively suggest that replication is required for vectors to reach peripheral ganglia following footpad inoculation. However, this problem can be overcome if sufficient pfu are used and vectors are administered directly into a nerve.

The results presented in figure 4.6 show that after injection of 1764 27-/4- pR19CMVlacZ, the numbers of lacZ positive neurons, determined by X-Gal staining, were consistent between 2days and 1 month p.i. Previously a similar vector had been constructed, 1764 pR19CMVlacZ (see section 3.2.2.1.), without ICP27 and ICP4 deleted, and assessed following sciatic nerve and footpad inoculation (see figure 3.2.). The replication competent vector gave high levels of gene delivery following sciatic nerve and footpad inoculation but results tended to be inconsistent between animals. The replication incompetent vector reported here, 1764 27-/4- pR19CMVlacZ gave highly consistent results between animals. This observation was possibly attributable to replication of the 1764 pR19CMVlacZ vector in the periphery (cells of the footpad or axonal bundle). This could function to increase vector toxicity and/or titre, and result in different amounts of vector potentially able to reach the DRG in different animals.

Previous work has reported that replication deficient vectors, whilst non-toxic, show a repression of transgene expression in cultured cells immediately after infection (Samaniego et al. 1998). This was suggested to be a result of the lack of expression of ICP0. The replication incompetent vector described here, 1764 27-/4- pR19CMVlacZ, expresses a transgene in the PNS for at least 1 month (Palmer et al. 2000), in the CNS for at least 1 month and in primary DRG cultures for at least 1 week (Lilley et al. 2001). A similar virus 1764 27-/4-/vhs-pR20.5, containing the pR20.5 cassettes in the vhs locus, was shown to express GFP and lacZ up to 3 weeks in organotypic hippocampal slice cultures and for 28 days in cultured Vero cells (Lilley et al. 2001). These findings, when compared to other vectors deficient in IE gene expression, suggest that elements of the LATP2 region included in the promoters used, are responsible for maintenance of transgene expression. Alternatively as the ICP0 gene has
not been deleted, residual levels of ICP0 expression may aid in the continued gene expression observed.

In conclusion work performed in this section has successfully identified a HSV-1 vector that is capable of both lytic and latent transgene expression in the PNS that is persistent and consistent over time. Previously (see chapter 3), successful transgene expression was achieved during acute infection but which declined rapidly during latent infection. The result seen collectively in chapters 3 and 4 suggest that long term expression can be achieved by a lack of IE gene expression in the vectors. These genes have previously been shown to be cytotoxic. It is likely that the increase expression observed with these replication incompetent vectors is due to a reduced cytotoxicity and/or the lack of immune response to the vector. However the exact reasons remain to be seen.
CHAPTER 5:

STUDIES TO DEVELOP INDUCIBLE TRANSGENE EXPRESSION SYSTEMS USING REPLICATION COMPETENT VECTORS
CHAPTER 5:
STUDIES TO DEVELOP INDUCIBLE TRANSGENE EXPRESSION SYSTEMS USING REPLICATION COMPETENT VECTORS.

5.0. INTRODUCTION.
Regulatable gene expression has been extensively studied over the past decade. In order to avoid the pleiotrophic effects of gene expression that are associated with endogenous eukaryotic promoters and enhancers, most of the successful systems now used in eukaryotic animal models rely on prokaryotic regulatory elements (Gingrich and Roder 1998). The ability to control gene expression provides a means by which physiological processes throughout development and cytotoxic gene function can be studied.

Regulated gene delivery would be highly advantageous and theoretically improve many gene therapy protocols since the inappropriate excess or lack of a delivered transgene is often intolerable. For example the excess or lack of insulin in diabetic patients will cause hypoglycaemia or hyperglycaemia respectively. Successful systems making use of prokaryotic regulatory elements include the 'Tet-Off' and 'Tet-On' systems (Gossen and Bujard 1992; Gossen et al. 1995), the ecdysone system (No et al. 1996) and a progesterone antagonist (RU486) system (Wang et al. 1994). The construction of regulatable HSV-1 vectors would provide potentially an ideal system for inducible gene therapy protocols in the nervous system. Such a vector would ideally have low basal expression in the uninduced state, have rapid induction kinetics that can be quickly and efficiently reversed, should not interfere with endogenous factors and the ligand should be safe, easy to administer and available to neurons. Systems such as this would not only greatly enhance the safety profile of gene therapy protocols but would likely provide an improved means to study gene function in the nervous system.

Previously there have been a few reported ligand regulatable HSV-1 vectors. These vectors are amplicon based tetracycline regulated (Tet-Off) vectors (Ho et al. 1996; Fotaki et al. 1997) and a disabled mifepristone (RU486) inducible vector (Oligino et al. 1998). The efficacy of these vectors was tested only in the
CNS. As yet there have been no reports of a ligand inducible HSV vector in the PNS. The 'Tet-Off' amplicon vectors contained both elements of the regulatory system, the transcriptional transactivator (tTA) and the tetracycline response element (TRE) in the same amplicon. These vectors achieved a 10fold (Fotaki et al. 1997) and 60fold (Ho et al. 1996) repression of reporter gene activity in vivo upon administration of doxycycline or tetracycline respectively. Whilst these reports provided proof that a tet regulated HSV-1 vector could be constructed, they also highlighted drawbacks of the system. Gene expression rapidly declined between 2 days and a week, probably as a result of the mixed population of helper virus and amplicon vector. Thus characterisation of the system beyond this time could not be studied. In addition (and in both cases), there was significant basal reporter gene expression in the uninduced state, e.g. $442 \pm 83$ lacZ positive neurons in the off state (Fotaki et al. 1997). Such problems need to be overcome in order for the system to be of significant use.

The mifepristone inducible vector achieved a 150fold induction of β-galactosidase expression in vivo. However as with the 'Tet-Off' amplicon vectors, the system was only characterised over a short period (48 hours). Encouragingly, in this report the levels of β-galactosidase expression in the hippocampus of untreated animals (without ligand) was barely detectable above background.

The aim of the work in this chapter was to develop ligand inducible replication competent HSV vectors for use in the peripheral nervous system. Initially three regulatable plasmid constructs were created, each combining all the elements of the particular ligand inducible system into one expression cassette. The systems studied were the 'Tet-On' system (Gossen et al. 1995), the ecdysone system (No et al. 1996) and the mifepristone system (Wang et al. 1994). Recombinant HSV vectors were then constructed and tested ex vivo and in vitro.
Chapter 5

5.1. MATERIALS AND METHODS.

5.1.1. Assessment of Ligand Inducible LacZ Activity in Plasmid Constructs.

Transient transfection assays (see section 2.2.4.1) were performed with the ligand inducible cassettes, see later. 5μl of Midiprep DNA (Qiagen, Chatworth, USA) was used per-transfection (see section 2.1.3.2.). 24hrs post transfection media was removed and new media added which either did or did not contain the appropriate ligand (see table 5.1). Media without ligand contained an equal volume of vehicle only. The transfection was then left for a further 24hrs before the cells were harvested and β-galactosidase activity determined via luminometry assay (see section 2.2.4.2), X-Gal staining (see section 2.2.4.3.) or FACS analysis (see section 6.1.2.).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
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<tbody>
<tr>
<td>Tetracycline (tet) (Sigma, Dorset, UK)</td>
<td>1mg/ml in 70% EtOH</td>
<td>1μg/ml</td>
</tr>
<tr>
<td>Doxycycline (dox) (Sigma, Dorset, UK)</td>
<td>1mg/ml in dH2O</td>
<td>2μg/ml</td>
</tr>
<tr>
<td>Muristerone A (mur A) (Invitrogen, The Netherlands)</td>
<td>0.5mg/ml in 100% EtOH (1mM)</td>
<td>10μg/ml</td>
</tr>
<tr>
<td>PonasteroneA (ponA) (Stratagene, UK)</td>
<td>1mg/ml in 100% EtOH (2mM)</td>
<td>10μg/ml</td>
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<tr>
<td>Mifepristone (RU 486) (Sigma, Dorset, UK)</td>
<td>1mM in 80% EtOH</td>
<td>10μM (1 in 100 dilution of stock, 10μl/ml)</td>
</tr>
</tbody>
</table>

Table 5.1. Ligand stock solutions and working concentrations used in transient transfection assays and in vector purification.

5.1.2. Ex Vivo Characterisation of Ligand Inducible Expression Vectors.

Mice were inoculated unilaterally via sciatic nerve injection with 2-5μl of ligand regulatable expression vector at appropriate doses. 2days post inoculation DRG
were removed and placed immediately in FGM containing 100µg/ml Acyclovir (Zovirax, Wellcome) with or without ligand (see table 5.2.). DRG were left for 24hrs at 37°C/5%CO₂ and then removed and fixed for 1hr on ice in 4% PFA in 1XPBS. The DRG were then washed 3 times with 1X PBS for 15mins each wash. The DRG were then placed in 100µl of DRG X-Gal solution (5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆.6H₂O, 1mM MgCl₂, 0.02% sodium deoxycholate, 0.02% NP-40, and 40mg/ml X-Gal [dissolved in DMSO] in 1 x PBS) and incubated at 37°C overnight. The X-Gal solution was then removed and the DRG placed in 70% v/v glycerol and stored at 4°C prior to photography.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
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</thead>
<tbody>
<tr>
<td>Doxycycline (dox)</td>
<td>1mg/ml in dH₂O</td>
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<tr>
<td>(Sigma, Dorset, UK)</td>
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<td>PonasteroneA (ponA)</td>
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<td>20µg/ml</td>
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<tr>
<td>(Stratagene, UK)</td>
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</tr>
</tbody>
</table>

Table 5.2. Ligand stock concentrations and working concentrations for ex vivo vector analysis.

5.2. RESULTS.

5.2.1. A Single Cassette can Incorporate Multiple Elements of a Ligand Inducible Expression System.

Most available ligand inducible expression systems are based on the use of two plasmids, one plasmid containing the response element and gene of interest and the second plasmid containing the transcriptional transactivator. The transactivator protein binds to the ligand and interacts with the response element, the effects of which are either a repression or activation of the transgene, the effects of which are reversible. In cell culture systems of this sort, effectiveness is hampered by the fact that in order for the system to work efficiently, introduction of both plasmids into the same cell and at the correct ratio is required which cannot be precisely controlled. Thus incorporation of the response element and transactivator into the same plasmid would potentially be
advantageous in facilitating work *in vitro*. In addition, in terms of vector production this would remove the need for a multi-step construction process. Such bicistronic expression cassettes have been described in chapter 3 (section 3.2.3), notably pR20.9 and pR20.5. These cassettes contain a central LATP2 region flanked on either side by two different promoters in a back-to-back orientation driving expression of the reporter genes, GFP and *lacZ*. Using the same strategy, three ligand inducible constructs were made expressing elements of the 'Tet-On' system, the ecdysone system and the mifepristone system.

5.2.2. Expression Cassettes and Cloning Strategies.

5.2.2.1. Tet-On-pR20.4.
Previously in the laboratory plasmid pR20.4/acZ was constructed. This plasmid contains a central LATP2 region (HSV nt 118866-120219, *PstI* to *BstX1*), see figure 5.1. Downstream to this is a CMV promoter (*MluI*-*HindIII* from pCDNA3; Invitrogen), and rtTA (reverse tetracycline controlled transcriptional transactivator, *EcoRI*-*BamHI* fragment from p17-1, obtained from H. Bujard). Upstream to LATP2 is TetR (tet repressor, *XhoI*-*EcoRI* from pUHD10-3, obtained from H. Bujard) and *lacZ*-pA (pCH110-Pharmacia). See figure 5.1. Here the pR20.4/acZ cassette was cloned into UL43 flanking regions at the unique *NsiI* site (HSV nt 94911 [in a BamHI-EcoRI fragment HSV nt 91619-96751 in pGem2]). See figure 5.1.

5.2.2.2. Ecdysone-pR20.8.
Previously in the laboratory plasmid pR20.8/acZ was constructed. This plasmid contains a central LATP2 region (HSV nt 118866-120219, *PstI* to *BstX1*), downstream to this is a CMV promoter (*MluI*-*HindIII* from pCDNA3; Invitrogen), VgEcR (*PstI*-*BstXI* from pVgRXR; Invitrogen), IRES (*EcoRI*-*NcoI* from pCITE-1; Professor J Almond, University of Reading, Reading, UK) and RXR (6631-8028 from pVgRXR; Invitrogen). Upstream to the LATP2 region is 5xE/GRE *P_{HSP} (2-511 from pIND; Invitrogen) and *lacZ*-pA (pCH110-Pharmacia). See figure 5.1. Here the pR20.8/acZ cassette was cloned into UL43 flanking regions at the
unique NsiI site (HSV nt 94911 [in a BamHI-EcoRI fragment HSV nt 91619-96751 in pGem2]). See figure 5.1.

5.2.2.3. Mifepristone-pR20.11.

Previously in the laboratory plasmid pR20.11GFP had been constructed. This plasmid contained a central LATP2 region (HSV nt 118866-120219, PstI to BstX1), downstream to this is a SpeI/BamHI fragment from pCEP4-GLVP(H), containing a CMV promoter and a chimeric regulator, GLVP (obtained from Yaolin Wang, Baylor College of Medicine, Texas). Upstream to LATP2 is a Xhol to HindIII fragment from p17x4TATA CAT, containing 4 copies of the GAL4 binding sequence, 17merx4 TATA (obtained from Yaolin Wang, Baylor College of Medicine, Texas). This drives expression of an E/GFP reporter gene (Clontech), see figure 5.1. Plasmid pR20.11lacZ was constructed here by digestion of pR20.11GFP with BamHI, removing the E/GFP+pA, replacing this fragment with a BamHI fragment from pPIL19G (previously constructed lab plasmid) containing lacZ-pA. pR20.11lacZ was cloned into UL43 flanking regions at the unique NsiI site (HSV nt 94911 [in a BamHI-EcoRI fragment HSV nt 91619-96751 in pGem2]). See figure 5.1.
Figure 5.1. Vector backbone and ligand inducible expression cassettes. A. 1764 vector backbone deficient in ICP34.5 and VP16. Expression cassettes were inserted at the UL43 locus B. pR20.4lacZ, the 'Tet-On' expression cassette. C. pR20.8lacZ, the ecdysone inducible expression cassette. D. pR20.11lacZ, the mifepristone inducible expression cassette. LacZ, β-galactosidase. CMV, cytomegalovirus promoter. LATP2, HSV latency associated promoter (HSV nt 118866 to 120219). TRE, tetracycline response element. rTtA reverse tetracycline controlled transcriptional transactivator. 5xEGRE, Ecdysone/Glucocorticoid response element. P_{ΔHSP}, heat shock minimal promoter. VgEcR, VP16/ecdysone receptor fusion. RXR, Retinoid-X-Receptor. GLVP, a chimeric protein consisting of the ligand binding domain of the human progesterone receptor, a yeast GAL4 binding domain and a VP16 transactivator domain. 17merx4 TATA, 4x GAL4 DNA binding sites.
5.2.3. Transient Transfection Assays of Ligand Inducible Expression Cassettes.

To test the efficacy of the ligand inducible cassettes pR20.4/UL43, pR20.8/UL43 and pR20.11/UL43, transient transfection assays were performed (see section 5.1.1.). Duplicate transfection were performed for each expression cassette. For each duplicate transfection the cassettes were incubated with or without ligand, as described in section 5.1.1. These transfections were repeated four times. Three sets were analysed using a luminometry assay kit (Galacto-Light™ β-galactosidase Reporter Gene Assay System [Tropix, Bedford, MA, USA]), see section 2.2.4.2) and the last set was fixed and analysed following X-Gal staining (see section 2.2.4.3.). The pR20.4 cassette was analysed twice, once using tetracycline as the induction ligand and the second time using doxycycline as the ligand. It has been reported previously that doxycycline works to more efficiently transcriptionally activate the ‘Tet-On’ system than tetracycline or other tetracycline derivatives (Gossen et al. 1995).

Figure 5.2 shows the results of X-Gal staining of the transient transfections. It is clear that the pR20.4 (Tet-On) and the pR20.11 (Mifepristone) cassettes display high basal activity in the absence of corresponding ligand. In both cases addition of ligand to the transfection appeared to make no observable difference in β-galactosidase expression. Results in figure 5.3 show β-galactosidase activity as determined by luminometry assay. Results are displayed as the average of the three transfections. Here results show that the pR20.4 cassette displays a 4.2-fold and 1.3-fold lacZ induction using doxycycline and tetracycline respectively. The pR20.11 cassette displays a 2.7-fold induction upon addition of RU486. Collectively, staining and enzyme assay show that whilst these cassettes are slightly responsive to ligand as measured by luminometry assay they are also "leaky". Transcriptional activation of the CMV and ΔHSP promoters, downstream to the response elements in the ‘Tet-On’ and mifepristone cassettes respectively, appears to be independent of the presence of the ligand.

In the case of the pR20.8 cassette, X-Gal staining of the transient transfection shows that in the absence of ecdysone few lacZ positive cells can be observed.
Upon addition of 10 μg/ml of ecdysone to the transfection the number of lacZ positive cells increases. Luminometry assay analysis of the transfection show that the pR20.8 cassette gives a 25-fold induction upon addition of ligand. The pR20.8 cassette thus appeared to be the most effective of the three regulatable systems tested at giving inducible gene expression.

It is speculated that the reason for the high basal activity of the cassettes may be due to elements in LATP2 or CMV which might act to increase the basal activity of the TRE, GRE or the TATA promoters in pR20.4, pR20.8 and pR20.11 respectively. However, if this is the case, the effect seems to be less marked in the pR20.8 cassette, which has provided the most promising results so far.
Figure 5.2. In vitro analysis of ligand inducible expression cassettes. Duplicate transient transfections of each plasmid were performed on BHK cells. 24 hours post transfection fresh media was added that contained either ligand or vehicle only. 48 hours post transfection media was removed and cells were fixed and X-Gal stained to determine β-galactosidase activity. 

A. pR20.4lacZ, 'Tet-On' inducible cassette with or without 2μg/ml doxycycline.

B. pR20.4lacZ, 'Tet-On' inducible cassette with or without 1μg/ml tetracycline.

C. pR20.8lacZ, ecdysone inducible cassette with or without 10μg/ml muristeroneA.

D. pR20.11lacZ, mifepristone inducible cassette, with or without 10μm RU486.
Figure 5.3. Inducible transactivation of β-galactosidase after transient transfection of ligand inducible expression cassettes into BHK cells. Enzyme activity was measured using a Galacto-Light™ β-galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA). A. pR20.4 ‘Tet-On’ construct either with or without 2µg/ml doxycycline or 1µg/ml tetracycline. B. pR20.8 ecdysone construct either with or without 10µg/ml muristeroneA. C. pR20.11 Mifepristone construct either with or without 10µm RU486. 5µl of midi-prep plasmid DNA was transfected into BHK cells in duplicate. 24 hours post transfection new media was added either with or without ligand. 48 hours post transfection cells were harvested and assayed for β-galactosidase activity following protocol 2.2.4.2. Data represents the average figures from three sets of transfections. Error bars represent a standard deviation of 1.
A. Tet-On Tetracycline Regulatable

![Graph showing β-Galactosidase Activity]

- Dox+ vs. Dox-
- Tet+ vs. Tet-
- 4.2 fold increase in Dox+ compared to Dox-
- 1.3 fold increase in Tet+ compared to Tet-

B. pR20.8 Ecdysone Regulatable

![Graph showing β-Galactosidase Activity]

- PA+ vs. PA-
- 24.9 fold increase in PA+ compared to PA-

C. pR20.11 Mifepristone Inducible

![Graph showing β-Galactosidase Activity]

- MP+ vs. MP-
- 2.7 fold increase in MP+ compared to MP-
5.2.4. Production of Tet-On, Ecdysone and Mifepristone HSV Vectors.
Transfections to produce vectors were performed following protocol 2.2.5. 1764 viral DNA was used in all cases. The 1764 virus strain is deficient in VP16 and ICP34.5 and has been described previously, see section 2.2.1. (Coffin et al. 1996). Prior to transfection the pR20.4lacZ/UL43 plasmid was linearised with XmnI (XmnI cuts in pGem2) and the pR20.8lacZ/UL43 plasmid was linearised using Muml (Muml cuts in UL43 flanking regions). The pR20.11lacZ/UL43 vector was not linearised due to an inability to find a restriction enzyme that did not cut in the pR20.11 cassette. After transfection recombinant plaques were identified by incubation of the harvested transfection with the appropriate ligand (see table 5.1). Plaque purification of recombinant vectors was performed as described in section 2.2.5.3. 3 plaques were purified for each different vector. At all stages of purification ligand was added to the media in order to select recombinant plaques (see table 5.1.). The 'Tet-On' vector was purified using doxycycline since the pR20.4 plasmid responded better to this ligand in transient transfection assay. The 'Tet-On' (1764 pR20.4lacZ/UL43) and ecdysone (1764 pR20.8lacZ/UL43) vectors were purified successfully, however, the mifepristone vector (1764 pR20.11lacZ/UL43) proved somewhat difficult to purify and thus purification was abandoned, (see section 5.2.5).

5.2.5. In Vitro Analysis of Vectors on Permissive Cells.
Plaque phenotypes of the 2 vectors, 1764 pR20.4lacZ/UL43 (Tet-On) and 1764 pR20.8lacZ/UL43 (ecdysone) can be seen in figure 5.4. BHK cells were infected with each virus and left for two days for plaques to appear. Vectors were either incubated with or without ligand and then X-Gal stained to determine β-galactosidase activity. It is apparent that within the pR20.4 vector lacZ expression from tetO/CMV occurs at high levels even in the absence of doxycycline. This was unsurprising considering the result seen following transient transfection of the pR20.4 plasmid. However, the phenotype of the pR20.8 vector was different when incubated with or without murA. In the absence of murA, lacZ activity is observed, however with the addition of murA lacZ activity appears to increase and plaques stain more strongly with X-Gal.
No quantitative experiments, such as luminometry, were performed on BHK cells infected with vector because the infections were intended only as a quick
look see and would be tested *in vivo* anyway. Since the pR20.8 plasmid produced low basal lacZ activity in transient transfection experiments, this result possibly indicates that in the context of the HSV genome, β-galactosidase expression may be activated by a viral protein.

The 1764 pR20.11/lacZ/UL43 vector proved difficult to purify since plaques were difficult to identify and were only stained faintly with X-Gal in about half the cells of each plaque. The lacZ expression phenotype was similar to that of the pR20.9 vectors described in chapter 3 (section 3.2.3.2.). In addition, the phenotype changed between rounds of plaque purification. Sometimes plaques that had previously stained faintly with X-Gal no longer stained at all during later rounds. For these reasons, plus the fact that the pR20.11 cassette had not performed well in transient transfection experiments, purification of this virus was abandoned.
Figure 5.4. Plaque phenotype of replication competent ligand inducible vectors on permissive BHK cells. A. 1764 pR20.4lacZ/UL43, 'Tet-On' inducible vector, with or without 2μg/ml doxycycline. B. 1764 pR20.8lacZ/UL43, ecdysone inducible vector, with or without 10μg/ml muristerone A.
5.2.6. The ‘Tet-On’ Vector Shows Poor Efficacy In Vivo.

Even though the pR20.4 vector appeared to have a low efficacy in vitro during purification, this ‘Tet-On’ vector 1764 pR20.4lacZ/UL43 was tested for efficiency in vivo following protocol 5.1.2. Briefly, four mice were injected unilaterally into their rear sciatic nerve with $1 \times 10^7$ pfu/ml of vector. 48 hours post inoculation DRG were extracted and incubated overnight in FGM containing 100μg/ml acyclovir (Zovirax, Wellcome) either with or without 4μg/ml doxycycline. 2 sets of DRG were incubated overnight with ligand and 2 sets without ligand. DRG were then X-Gal stained to determine β-galactosidase activity. Acyclovir was added to the overnight incubation media since explantation of virally infected DRGs is known to cause reactivation or replication competent virus and thus production of viral proteins (Stevens and Cook 1971), which might otherwise affect tet regulated gene expression.

Results can be seen in figure 5.5. It can clearly be seen that in the absence of the transactivating ligand, doxycycline, β-galactosidase activity is high and comparable with those DRG incubated with doxycycline. These results are consistent with the in vitro appearance of 1764 pR20.4lacZ/UL43, which produced viral plaques on permissive BHK cells that were strongly blue with or without doxycycline.

Due to these disappointing but somewhat expected results, no further work was performed using the 1764 pR20.4 ‘Tet-on’ vector.
"Tet-On" Vector
1764 pR20.4lacZ/UL43

-Doxycycline

+Doxycycline

Figure 5.5. Ex Vivo analysis of a "Tet-On" replication competent vector. Mice were inoculated unilaterally into the sciatic nerve with 2-5μl of 1x10^7 pfu/ml of 1764 pR20.4lacZ/UL43. 2 days post inoculation DRG were extracted and incubated for 24 hours in media plus 100μg/ml acyclovir, either with or without 4μg/ml doxycycline. DRG were then fixed and X-Gal stained to determine β-galactosidase activity.
5.2.7. Characterisation of the Ecdysone Inducible Vector.

The 1764 pR20.8lacZ/UL43 vector was characterised by western blot detection for the RXRα protein. In the ecdysone inducible expression system Retinoid-X-Receptor (RXR) forms a heterodimer with the VgEcR protein (a chimeric ecdysone receptor) to form a complex which is the transactivator (No et al. 1996). The transactivator binds to ecdysone response elements to activate gene transcription from a downstream promoter. In the pR20.8 cassette the RXR gene is downstream to an IRES sequence (see figure 5.1).

BHK cells were grown to 90% confluency in 35mm dishes and infected at an MOI of 0.1 with vectors A. 1764pR20.5/UL43Cre (see section 7.2.2.) and B. 1764 pR20.8/UL43. Vector A was a negative control for RXRα expression and was chosen because it was otherwise very similar to the 1764pR20.8lacZ/UL43 vector (see figures 5.1 and 7.3.). 24 hrs post infection cells were harvested and samples prepared for western blot analysis following protocol 2.2.7.2 (see chapter 2). Approximately 20μl of each sample was run on a 10% acrylamide/10% SDS gel containing 2M urea. Results can be seen in figure 5.6. Results show that the pR20.8lacZ/UL43 vector expresses a 56kDa RXRα protein as expected, whereas the negative control vector did not.
Figure 5.6. Western blot detection of retinoid-X-receptor (RXR) expression from a replication competent vector. BHK cells were grown to 90% confluency and were infected at an MOI of 0.1. 24 hours post infection cells were harvested and samples prepared. Approximately 20μl of each sample were run on a 10% acrylamide/10%SDS gel containing 2M urea, alongside 5μl of molecular weight markers. A. 1764pR20.5/UL43Cre, negative control vector not expressing RXRα (see section 7.2.2.). B. 1764pR20.8/lacZ/UL43, ecdysone inducible vector (see section 5.2.2. Detection with RXRα antibody (Santa-Cruz Biotechnology) reveals a 56kDa protein only in the 1764 pR20.8/lacZ sample, indicating that the virus expresses RXRα.
5.2.8. The Ecdysone Inducible Vector Shows Limited Efficiency in Vitro.

The 1764 pR20.8/lacZ/UL43 vector was purified successfully and a high titre stock was grown for experimental purposes. In the absence of an antibody that could reliably detect the VgEcR protein the 20.8/lacZ/UL43 vector was verified in vitro for inducible lacZ expression. To test the vector in vitro BHK cells were grown to 90% confluency in 35mm dishes and infected in duplicate with various different pfu of vector, $10^2$, $10^3$ and $10^4$. Cells were left for 48 hours at $37^\circ$C/5%CO$_2$ and duplicate wells were incubated either with or without 10μg/ml ponA. Infected cells were then harvested and enzyme activity was measured using a Galacto-Light™ β-galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA). Samples were diluted 1:10 before assay. Results can be seen in figure 5.7. The figure shows that at all pfu tested that the β-galactosidase activity increases upon addition of ponA, however the fold induction observed is negligible and it is difficult to conclude that the vector is significantly ligand responsive. After X-Gal staining of a duplicate set of identical experiments (results not shown) it was apparent that there was no perceptible difference between that lacZ activity when the virus was incubated with or with ponA, as observed by X-Gal staining. Basal levels of lacZ expression from the E/GREΔHSP were high. This was an expected result since during vector purification viral plaques had previously been found to stain strongly without the addition of ligand.

The pR20.8 cassette produced 25fold β-galactosidase induction with low basal leakiness following transient transfection into BHK cells. Results shown here therefore suggest that the loss of ligand mediated gene regulation from E/GREΔHSP is a result of the HSV vector. This could be the direct result of a HSV-1 protein. Or, since the transcriptional state of the cell is altered upon infection with most viruses, it could be speculated that infection of cells with a HSV vector would influence the transcriptional regulation in an indirect manner, resulting in increased lacZ expression.
Figure 5.7. *In vitro* Inducible transactiviation of β-galactoidase in the replication competent vector pR20.8lacZ/UL43. Enzyme activity was measured using a Galacto-Light™ β-galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA). LacZ activity is measured in arbitrary light units. BHK cells were grown to 90% confluency in 35mm dishes and cells were infected with virus at the indicated pfu. Cells were incubated for 48 hours in media with or without 10μg/ml ponasteroneA. Cells were then harvested and prepared for luminometry assay (see section 2.2.4.2.).
5.2.9. Efficacy of the Ecdysone Inducible Vector Ex Vivo.

It was speculated that replication of the 1764 pR20.8/lacZ/UL43 vector in vitro and thus production of viral proteins, might be a reason for the leakiness of lacZ gene expression from the pR20.8 cassette. This vector is deleted for both copies of the neurovirulence factor ICP34.5, which means that it cannot replicate in non dividing cells such as neurons (MacLean et al. 1991a). Thus, if the vector was to be tested in DRG following peripheral administration, then theoretically no replication induced gene expression should occur.

2-5μl of 2 x10⁶ pfu/ml of the vector was injected unilaterally into mouse sciatic nerve. DRG were extracted 48hrs post inoculation and incubated overnight in FGM media containing 100μg/ml acyclovir (Zovirax, Wellcome), either with or without 20ug/ml ponA. DRGs from 5 animals were incubated in media containing ponA, DRGs from 4 animals were incubated in media without ponA. The concentration of ponasteroneA used here was twice that recommended for transient transfection assays (see section 51.1.). As previously, acyclovir was added in order to prevent viral replication. After 24hrs incubation DRG were fixed, washed and X-Gal stained following protocol 2.2.8.3. The numbers of lacZ positive cells in L4 and L5 of each animal were counted. Results can be seen in figure 5.8A and quantified in 5.8B. Data shows that following sciatic nerve inoculation of mice with vector 1764 pR20.8/lacZ/UL43, and subsequent DRG explantation, the vector appears responsive to ponA mediated lacZ transactivation. There is a clear difference between the number of lacZ positive cells in those DRG incubated with ponA and those DRG incubated without ponA. These results would agree with the hypothesis that replication of the vector and thus a HSV-1 protein, contribute to transactivation of the lacZ gene from E/GREΔHSP in BHK cells in the absence of ligand.
Figure 5.8A. Inducible transactivation of β-galactoidase in the replication competent ecdysone regulatable vector pR20.8lacZ/UL43. Mice were injected unilaterally in the sciatic nerve with 2-5 μl of 2x10⁶ pfu/ml of vector. 48 hours post inoculation DRG were extracted and incubated overnight in FGM containing 100 μg/ml of acyclovir and either A. without ponA (4 animals) or B. with 20 μg/ml ponA (5 animals). DRG were then fixed and X-Gal stained to determine β-galactosidase activity.
Figure 5.8B. Inducible transactivation of β-galactosidase in the replication competent ecdysone regulatable vector pR20.8lacZ/UL43. Mice were injected unilaterally in the sciatic nerve with 2-5 μl 2x10^6 pfu/ml of vector. 48 hours post inoculation DRG were extracted and incubated overnight in FGM containing 100 μg/ml acyclovir and either with (animals 1-5) or without (animals A-D) 20 μg/ml ponA. DRG were then fixed and X-Gal stained. LacZ positive neurons in L4 and L5 of each animal were counted and the numbers added together as a measure of β-galactosidase activity.
5.2.10. A Replication Competent Vector Causes Induction of a Ligand Inducible Response Element in the Absence of Transactivator or Ligand.

Since it was possible that the expression of HSV proteins was the likely cause of leaky transactivation of the ecdysone response element and thus expression of *lacZ* from ΔHSP, it was decided to investigate this possibility. pIND (Invitrogen, The Netherlands) is a plasmid containing an ecdysone /glucocorticoid response element (E/GRE) upstream to a minimal heat shock promoter (ΔHSP) which drives expression of a *lacZ* gene (see figure 1.13). Transcription of *lacZ* should only occur in the presence of the VgEcR/RXR heterodimer and ligand such as murA or ponA (see figure 1.13). VgEcR is a fusion between the ecdysone response element (EcR) and the VP16 transactivation domain. RXR refers to the retinoid -X-receptor. The VP16 transactivation domain functions to activate the heat shock promoter when the complex is bound to E/GRE. It was speculated that activation of the ΔHSP was occurring in the absence of VP16 transactivation, either by exogenous VP16 from the virion tegument or by other HSV proteins such as ICP0 or ICP4. ICP0 is known to be a promiscuous transactivator of HSV and non-HSV promoters ((Everett 1985; Mosca et al. 1987; Nabel et al. 1988; Cheung et al. 1997; Samaniego et al. 1998). ICP4 functions to transactivate early and late HSV-1 gene expression (DeLuca et al. 1985; DeLuca and Schaffer 1985a). In addition ICP4 is known to transactivate non-viral gene promoters including the alpha and beta globin promoters (Cheung et al. 1997; Everett 1985). The vector used in this study 1764 pR20.8lacZ/UL43 was inactivated for VP16 transactivation by a 12bp insertion in the C-terminal transactivation domain (Ace et al. 1989). Thus it was thought unlikely that virion associated VP16 was the candidate gene interacting with E/GREΔHSP to produce leaky lacZ expression.

Transient transfection of 5μl of pIND was performed into either BHK or 27/12/M:4 cells following protocol 2.2.4.1. 24hrs post transfection cells were superinfected with viral vectors at an MOI of 1. A mock superinfection was also performed on BHK cells. A list of the vectors used can be seen in table 5.3. Vectors were either 'white' and contained no transgene or contained a GFP transgene. Vector 1764 27-/P2-/ MSV/GFP contained a MSV/GFP cassette in
vhs but was otherwise the same as described previously (section 4.1.1.1. (Lilley et al. 2001). Vector 1764 27-/P2-/4- MSV/GFP contained an MSV/GFP cassette in ICP4 but was otherwise the same as described previously (section 4.1.2.1 (Lilley et al. 2001)) Vector 1764 27-/P2-/4- MSVGFP was used to superinfect BHK and 27/12/M:4 cells, whereas the other vectors were only used to superinfect BHK cells. BHK cells are not permissive for growth for the 1764 27-/P2-/4- MSV/GFP vector whereas 27/12/M:4 cells complement ICP24 and ICP27 gene deletions and are thus permissive (see section 2.2.2.3.). 24hrs post superinfection cells were fixed and X-Gal stained to determine β-galactosidase expression. Results can be seen in figure 5.9.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Genes deleted /inactivated</th>
</tr>
</thead>
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<td>17+ 27-</td>
<td>ICP27 deleted (Howard et al. 1998)</td>
</tr>
<tr>
<td>1764</td>
<td>ICP34.5 deleted, VP16 inactivated (Coffin et al. 1996)</td>
</tr>
<tr>
<td>1764 27-/P2-/vhs-MSV/GFP</td>
<td>ICP34.5 deleted, VP16 inactivated, ICP27 deleted, LATP2 deleted, vhs inactivated (Lilley et al. 2001).</td>
</tr>
<tr>
<td>1764 27-/P2-/4-MSVGFP</td>
<td>ICP34.5 deleted, VP16 inactivated, ICP27 deleted, LATP2 deleted, ICP4 deleted (Lilley et al. 2001).</td>
</tr>
</tbody>
</table>

**Table 5.3. Viral vectors used in superinfection experiments.** Details of gene deletions and inactivations are given. All vectors are based on the 17+syn strain (Brown et al. 1973).

Results in figure 5.9 show that in BHK cells only the fully replication incompetent HSV-1 vector, 1764 27-/P2-/4- MSVGFP does not transactivate β-galactosidase expression from E/GREΔHSP. Levels of lacZ expression appear similar to those observed when a transfection of pIND alone was performed, figure 5.10A. However on 27/12/M:4 cells when this vector is replication competent, transactivation of E/GREΔHSP is observed. Previously in our laboratory it has been found that on non-complementing cells a 1764 27-/P2-/4- vector exhibits minimal levels of ICP0, ICP22 and ICP47 gene expression, even at high MOI, and that the only viral gene detected to significant levels was ICP6.
This would suggest that, as hypothesised, one of the HSV-1 IE proteins is responsible for transactivation of E/GREΔHSP.

The results presented here indicate that the only vector that does not transactivate an ecdysone inducible response element is one disabled for ICP27, ICP4 ICP34.5/OrfP, VP16, and LAT2. Replication incompetent vectors of this nature are known to produce minimal levels of IE genes in non-complementing cells, the only HSV protein produced to significant levels is ICP6 (Lilley et al. 2001). Thus it would sensible to construct an ecdysone inducible vector in a fully replication incompetent backbone in order to minimise transactivation of E/GREΔHSP by a viral protein. This work can be seen in chapter6.
Figure 5.9. Transactivation of an ecdysone response element by HSV vectors. Transient tranfection of 5µg of pIND (Invitrogen) was performed on BHK and 27/12/M:4 cells. 27/12/M:4 cells are a BHK derived cell line complementing HSV genes ICP27, ICP4 and VP16. (see section 2.2.2.3). pIND is a plasmid containing a hybrid ecdysone response element (5xER/GRE) which activates transcription of a lacZ gene from a minimal heat shock promoter (ΔHSP). Transactivation should only occur in the presence of a RXR/VgEcR heterodimer and a ligand e.g. ponasreroneA. (see section 1.6.3.) 24 hours post transfection cells were infected with different vectors at an MOI of 1. 24 hours post infection cells were fixed and X-Gal stained to determine lacZ activity. A. pIND plasmid alone. B. pIND plus vector 17+27-. C. pIND plus vector 1764. D. pIND plus vector 1764/P2-/27-/vhs-MSV/GFP. E. pIND plus vector 1764 27-/P2-/4-/MSV/GFP. 17+ is a HSV-1 strain isolated in Glasgow, 17syn+ {Brown, Ritchie, et al. 1973 1914 /id} ICP27- refers to a deletion in the IE gene ICP27 (see section 4.1.1.1.). ICP4- refers to a deletion in the IE gene ICP4 (see section 4.1.2.1.). 1764 refers to a virus strain deleted for ICP34.5 and inactivated for VP16 {Coffin, MacLean, et al. 1996 1381 /id}. P2- refers to a deletion in the endogenous LAT region (HSV nt 118768-120470). vhs- refers to inactivation of the virion host shut off protein, vhs (see section 4.1.1.1.).
BHK 27/12/M:4

A. pIND

B. pIND 17+ 27-

C. pIND 1764

D. pIND 1764 27-/P2-/vhs- MSV/GFP

E. pIND 1764 27-/P2-/I4- MSV/GFP
5.3. DISCUSSION.
The ability to precisely control gene regulation will be a valuable tool for many
gene therapy protocols. As yet, systems of this type have suffered largely from
high basal expression of the reporter gene in the uninduced state and low
induction levels. Therefore new developments should be aimed at improving
these aspects of the system.

This study describes the development of replication competent ligand inducible
HSV vectors. Optimisation of gene delivery to the PNS using replication
competent vectors was concurrently being undertaken (see chapter 1). The aim
here was to use this information to allow construction of a regulatable HSV-1
vector. In this study three different systems were compared, a tetracycline
inducible (‘Tet-On’), a steroid hormone inducible (ecdysone) and a
progesterone antagonist inducible (mifepristone) system. HSV-1 vectors were
constructed containing elements of each system and tested for their efficacy in
vitro and ex vivo. The ecdysone inducible system, using the ecdysteroids
muristeroneA or ponasteroneA, was found to be the most efficient system
tested here both in vitro and ex vivo. In vivo studies have yet to be performed
due to shortages in the supply of ponA (see later). This work provides the first
reported evidence of both a regulatable HSV-1 vector suitable for use in the
peripheral nervous system and an HSV-1 ecdysone inducible vector.

Initial work involved testing of single expression cassettes that incorporated all
the elements of each regulatory system. The three expression cassettes were
p20.4 (Tet-On), pR20.8 (ecdysone) and pR20.11 (mifepristone). The cassettes
were cloned into UL43 flanking regions in order to generate a disabled HSV-1
vector. UL43 was chosen as the insertion site as it is a non-essential gene
(MacLean et al. 1991b). In addition earlier work has shown that transgene
expression from cassettes inserted within this locus were efficient both during
acute and latent infection (see section 3.2.3). The ‘Tet-On’ system was chosen
in favour of the ‘Tet-Off’ system (where gene expression occurs only in the
absence of tetracyclines) since this was a more direct comparison between the
ecdysone and mifepristone systems. Furthermore in terms of gene therapy
protocols a system whereby induction of gene expression is dependent upon
administration of a ligand (Tet-On) is preferable to a system whereby the continual presence of ligand is required in order to maintain a repressed state (Tet-Off). Indeed the properties of the ‘Tet-On’ system are of particularly advantage for in vivo studies whereby activation of a target gene does not depend on the half-life (clearance) of the effector.

Transient transfection assays of cassettes on BHK cells showed that only the pR20.8lacZ/UL43 cassette gave reasonable levels (25fold) of ligand inducible β-galactosidase expression. In comparison, the ‘Tet-On’ and mifepristone cassettes gave less than 5fold-lacZ induction upon addition of ligand. The low basal activity of the ecdysone inducible cassette was only marginally above that of background activity observed in mock-transfected cells. At this point it is unclear as to whether the off state is completely tight or whether the low basal activity observed would increase accordingly with the addition of more plasmid. It would have been sensible to repeat these transfections but using increasing volumes of plasmid DNA. In relation to the tet regulatable systems it has been suggested (Gossen and Bujard 1995) that results of transient transfection assays cannot be compared to results from stable cell lines which were shown to have gene repression up to 3 orders of magnitude. Generation of efficient stable cell lines rely on screening of many colonies in order to find a highly regulatable clone. In the generation of stable cell lines low basal activities can be achieved by plasmid integration into transcriptionally silent regions of the chromosome. Obviously this will not occur in transient transfections. However, it would be expected that a higher degree of regulation would be achieved following transient transfection assay of the ‘Tet-On’ pR20.4 construct, as an indication of efficacy. In conclusion the transient transfections show that the pR20.8lacZ/UL43 plasmid was likely to provide that most promising results when inserted into a HSV vector.

It has been reported previously that the efficacy of the ‘Tet-Off’ system is influenced by cell type (Ackland-Berglund and Leib 1995). In this report it was found that following transient transfection of plasmid, basal transcriptional activity of tetOCMV, in the absence of transactivator (tTA) and tetracycline and thus in the off state, varied greatly between cell lines. Transiently transfected
BHK cells were shown to have up to a 270-fold higher basal luciferase activity than stable HeLa or PC12 cells. In addition, BHK cells that stably expressed tTA and were transiently transfected with tetOCMV showed no luciferase regulation upon addition or removal of tet. In addition, the efficacy of a 'Tet-Off' retroviral vector has also shown to be cell type specific (Paulus et al. 1996). Since this response element, tetO/CMV, is common to both 'Tet-On' and 'Tet-Off' systems, it is possible that the high basal $\beta$-galactosidase activity observed following transient transfection assay of pR20.4lacZ/UL43 is due to the BHK cells used. Here it would have been sensible to use different cell lines such as HeLa cells or ND7 cells for in vitro studies. However, the creators of the tetracycline expression system argued that the results obtained in the transient transfection assays of Ackland-Berglund et al (Gossen and Bujard 1995) were not comparable with their findings of low basal transcription in selected clones of HeLa cells stably expressing tTA and tetO/CMV.

Since the pR20.11lacZ/UL43 and pR20.4lacZ/UL43 plasmids were verified with restriction digest analysis alone, results here do not preclude the possibility that generation of the plasmid had created an undetected mutation thus accounting for the lack of regulation observed, although this would seem unlikely. The use of western or northern blot analysis to detect rtTA (tetracycline reverse transactivator) or GV-LP (mifepristone transactivator) to examine this possibility would clarify this point. A more likely explanation is that juxtaposition of the regulatable promoters to LATP2 (in order to facilitate long term expression), increased basal activity of the promoters through the now reported enhancer effects of the region (Berthomme et al. 2000; Palmer et al. 2000). As a point of interest, western blot analysis was performed to detect rtTA from the pR20.4lacZ/UL43 plasmid and also the subsequent virus. However, despite repeated attempts the procedure was not successful, even in detecting the tet repressor in a sample from a known positive control. It is hypothesised that the tet-repressor antibody (BD-clontech, UK) was the problem. A further attempt should be made using and antibody from another source.

Despite the somewhat disappointing results of the 'Tet-On' and mifepristone plasmid constructs, all three expression cassettes pR20.4lacZ/UL43,
pR20.8lacZ/UL43 and pR20.11lacZ/UL43 were used to construct HSV-1 recombinant vectors. The 'Tet-On' and ecdysone inducible vectors were made and purified successfully. However, the recombinant mifepristone vector proved somewhat difficult to generate. Only after repeated transfection were recombinant plaques detectable. Furthermore during the purification process expression of β-galactosidase was very low, appeared unresponsive to mifepristone and was somewhat variable between rounds of purification (sometimes undetectable). For these reasons purification of this vector was abandoned. Previously a mifepristone inducible HSV-1 vector has been produced (Oligino et al. 1998). This was a double recombinant vector containing the RU486 transactivator and response element inserted into the tk and gC loci respectively. Here the vector was replication deficient due to a deletion in ICP4. The vector showed a 20fold induction of β-galactosidase expression in vitro and a 150fold induction of β-galactosidase in vivo upon administration of RU486. This data provides proof of principal that it is possible to generate a mifepristone inducible HSV-1 vector. In addition, as highlighted with the 'Tet-Off' system, BHK cells might be unsuitable for the generation and testing of the mifepristone system. Regulation of gene expression may be subject to interference by as yet unknown cellular factors.

Following the production of the 'Tet-On' vector it was tested in vitro on different cell lines, ND7, BHK and Vero cells, in order to establish the efficacy of ligand inducible β-galactosidase expression. The vector was tested on cell lines other than BHK cells in order to determine if regulation was cell type specific. Results of these experiments have not been shown, however there was no observable difference between the on and off states and lacZ expression was high in all cases and in all cells. The vector was also tested ex vivo following sciatic nerve inoculation of virus, explant of DRG and incubation overnight with doxycycline. As expected results show that even in the absence of doxycycline and thus the uninduced state, there is a high level of lacZ activity. Ideally the vector would have been tested in vivo by sciatic nerve inoculation of vector followed by i.p. injection of doxycycline or tetracycline. However, since ex vivo results indicated that the vector was ineffective in repression of basal lacZ activity, further in vivo analysis was not performed.
Subsequent to these results it was reported that HSV-1 infected cell proteins, ICP4, ICP0 and to a lesser extent ICP27 and VP16 influence tetracycline regulated transgene expression (Herrlinger et al. 2000). These findings, as well as the LATP2 enhancer function, might then explain lack of regulation seen from within a ‘Tet-On’ HSV-1 vector, even when on ND7 and Vero cells. The published work studied the ‘Tet-Off’ tetracycline regulatable system. Different HSV-1 mutants were used to superinfect Vero cells that had been: 1) transfected with a plasmid containing the luciferase gene under the control of tet operator sequences, tetO/CMV, 2) transfected with plasmids containing both tetO/CMV and the tetracycline transcriptional transactivator (tTA), or 3) infected with a HSV-1 amplicon expressing tetO/CMV and tTA. This showed that following transient transfection with tet-off constructs or infection with a tTA/tetOCMV-bearing HSV-1 amplicon, superinfection with a HSV-1 vector deregulated the ‘Tet-Off’ system. The HSV-1 mutant lacking ICP4 and VP16 (Johnson et al. 1994) appeared to interfere least with the elevation in baseline tetO activation. This vector was fully deleted for ICP4 and is reported to have had significantly reduced levels of ICP0. Other ICP4 vectors used in the study were reported to still express ICP0 (DeLuca et al. 1985; Samaniego et al. 1995). The group concluded that ICP4, ICP0 and to a lesser extent ICP27 and VP16 influence tetracycline regulated gene expression (Herrlinger et al. 2000). These results conflict slightly with comments by others (Fotaki et al. 1997), who suggested that VP16 in the virion of a helper virus does not interfere with tetracycline mediated repression in an amplicon in vivo. However, since the highest levels of lacZ repression observed here were only 10fold, this finding may not be significant.

The ecdysone inducible HSV vector showed a 1-2fold induction of β-galactosidase expression when tested on BHK cells. This was a disappointing result following those obtained from the transient transfection assays of the pR20.8lacZ/UL43 plasmid. However, when the same vector was tested ex vivo the vector was found to be clearly inducible as determined by counting of lacZ positive cells. Since the 1764 vector can replicate in BHK cells but should not replicate in DRG neurons, this phenomenon could be a result of non-replicating vector and a reduction in the amount of HSV-1 proteins available to mediate
transactivation. The superinfection experiments using pIND (ecdysone response element) and various viral vectors confirmed this hypothesis. Only a non-replication competent vector, deficient for ICP4, ICP27, ICP34.5 and VP16, did not transactivate the E/GREΔHSP response element in the absence of ligand and transactivator. This vector, 1764 27-/P2-/I4-MSV/GFP, had previously been shown to express minimal levels of ICP0, ICP22 and ICP47, even at high moi, and that the only viral gene detected to significant levels was ICP6 (Lilley et al. 2001). The vector 1764 27-/P2-/vhs- MSV/GFP caused transactivation of E/GREΔHSP. Since ICP4 is still functional in this vector, significant amounts of IE proteins, apart from ICP27, are expressed (Lilley et al. 2001). Collectively these results would suggest that it is one or more of the IE HSV-1 proteins that are responsible for transactivation of E/GREΔHSP in the absence of VgEcR and ponA. This situation, whereby HSV vectors expressing IE genes can transactivate the ecdysone responsive promoter, is similar to that found with HSV proteins and the tet regulatable system as reported by Herrlinger et al (Herrlinger et al. 2000). An obvious solution would thus be to produce a replication incompetent ecdysone inducible vector (see chapter 6) based on virus 1764 27-/P2-/I4- MSV/GFP.

One of the drawbacks of using the ecdysone system is the expensive price of the ecdysteroids, ponA and murA. In the experiments described here the in vivo analysis of the 1764 pR20.8/lacZ/UL43 vector was limited by the expense of the ligand ponA, since it was reported that doses of 20mg of ponA per animal would be required in order to achieve gene regulation (No et al. 1996). This was based on studies in transgenic animal models. More recent reports suggest that between 1-5mg of ponA per animal may be effective doses (Saez et al. 2000) and thus in vivo studies are now being considered.

High basal expression of a transgene in regulatable systems is a problem that needs to be addressed. Recently some groups have made use of chicken β-globin insulator elements to insulate transgene expression from the effects of upstream or downstream enhancers or promoters. Such elements can arise from within the viral vector itself, such as cis-acting elements in the LTRs, or from the cell genome following integration of retroviral vectors or plasmids.
Several groups have achieved mixed success using the β-globin insulator elements, for example in adenoviral vectors (Burcin et al. 1999a) (Steinwaerder and Lieber 2000) and transgenic mice (Wang et al. 1997). A common theme seen in these results is that whilst insulator elements may function in some circumstances to reduce basal gene expression, they can also act to reduce the overall levels of gene expression. Nevertheless, it is possible that the introduction of insulator elements into the regulatable cassettes seen here may be advantageous in reducing basal transgene expression.

In summary this section of work highlighted a steroid hormone inducible vector, 1764 pR20.8lacZ/UL43, as providing the most consistent transgene regulation in response to ligand. Basal gene expression is however still a problem that needs to be addressed. The vector also requires characterisation in vivo. Further development of this system, using multiple IE gene deficient vectors, is described in chapter 6.
CHAPTER 6:

STUDIES TO DEVELOP INDUCIBLE TRANSGENE EXPRESSION SYSTEMS USING REPLICATION INCOMPETENT VECTORS
CHAPTER 6: STUDIES TO DEVELOP INDUCIBLE TRANSGENE EXPRESSION SYSTEMS USING REPLICATION INCOMPETENT VECTORS.

6.0. INTRODUCTION.
In order to achieve efficient regulatable transgene expression in the context of an HSV vector it was speculated that a replication incompetent vector backbone not expressing HSV IE proteins will be required (chapter 5). It has been reported previously that HSV proteins can transactivate response elements and minimal promoters, both in the tetracycline and ecdysone responsive systems, in the absence of ligand or transactivator (Herrlinger et al. 2000) (see chapter5).

Replication incompetent HSV vectors are deleted for essential genes such that they cannot replicate unless these genes are provided in trans. A completely non-toxic HSV vector requires that no IE genes are expressed (Samaniego et al. 1998). Many different vectors have been constructed that are deleted for various combinations of IE genes (Wu et al. 1996; Samaniego et al. 1997; Preston et al. 1997; Krisky et al. 1998; Samaniego et al. 1998; Marshall et al. 2000; Lilley et al. 2001). Only one of these vectors is deficient in all of the IE genes (Samaniego et al. 1998). In our laboratory a vector has been constructed that is deleted for ICP4, ICP27, ICP34.5/OrfP and is inactivated for VP16 transactivation (see section 4.1.2.). On non-complementing cells the only viral protein produced at significant levels by this vector is ICP6 (Lilley et al. 2001). Thus, this virus backbone should be a suitable vector from which to create a ligand inducible vector, where basal gene expression levels are not affected by expression of IE genes.

The ligand regulatable expression cassettes described in chapter 5 contain the elements of each expression system located in a back to back orientation, such that gene expression occurs in different directions. It was hoped that positioning of the genes in opposite directions would lead to transcription enhanced in the long term by LATP2. Unfortunately only the ecdysone inducible cassette had low basal leakiness whereas the ‘Tet-On’ and mifepristone did not, for reasons previously discussed (see chapter 5). Other groups have taken different
approaches in the construction of regulatable vectors. Such approaches include the incorporation of all elements of the system into a single expression cassette and thus one vector (Hofmann et al. 1996; Paulus et al. 1996), by creating double recombinant vectors by separating each component of the system (Wang et al. 1997), or by using two separate vectors, one containing the transactivator the other the response element (Rendahl et al. 1998; Harding et al. 1998). Each group has met with different levels of success. The advantage of using two separate vectors is that they can be injected at different ratios in order to improve results. With a ‘Tet-Off’ vector a ratio of 1:20 transactivator to reporter vector was required in order to achieve efficient regulation (Harding et al. 1997). However, whilst this sort of system achieves good results, the use of one vector would be preferential since two vectors may not consistently infect the same cells and thus this would require the inoculation of higher vector doses.

Basal leakiness from minimal promoters is a consistent problem found with many regulatable systems. Fundamentally the problem arises because there is no physical block to transcription of the transgene in the uninduced state. Usually the transgene is located directly downstream to a minimal promoter and gene expression relies on the binding of a transactivator to the response element located upstream to the minimal promoter (Gossen and Bujard 1992; Wang et al. 1994; Gossen et al. 1995; No et al. 1996). Recently a novel system has been described in which tetracycline operator sequences (tetO) are located downstream to a minimal hCMV promoter (Yao et al. 1998). In this system and in the uninduced state the tet repressor binds to the tetO sequences and thus functions to repress expression of the transgene (see figure 1.12). Basal leakiness from the hCMV promoter cannot occur due to the presence of the TetR as a physical barrier to transcription. In the presence of tetracycline the TetR is prevented from binding to the tetO sequences and transgene expression occurs. This system theoretically has the advantages of the ‘Tet-On’ system inasmuch as transcriptional repression of the gene of interest is not dependent on the continual presence of ligand and activation of a target gene does not depend on the half-life (clearance) of the effector.
The aim of this section of work was to develop replication incompetent HSV-1 vectors for gene delivery to the PNS. It had already been shown that replication incompetent vectors were capable of efficient gene delivery to peripheral ganglia (see section 4.1.2). In light of the encouraging results seen in chapter 5, replication incompetent vectors were developed that contained the ecdysone regulatable system and tested in vitro, ex vivo and in vivo. The work reported in this chapter was undertaken in parallel to work in the later part of chapter 5. While at the end of chapter 5 construction of a multiply IE gene deficient vector containing pR20.8 would appear the next logical step, at the time of starting the work in this chapter a different approach was taken. The effects of the LATP2 promoter (Palmer et al. 2000) upon regulated gene expression were first investigated. In addition vectors were designed that would incorporate the TetR system.
6.1. MATERIALS AND METHODS.

6.1.1. In Vivo Characterisation of Ligand Inducible Expression Vectors.
Mice were inoculated unilaterally via sciatic nerve injection with 2-5μl of ligand regulatable expression vectors at appropriate doses. 2days post inoculation mice were injected IP in the ipsilateral side with 2-5mg of ponA. PonA was dissolved in 50μl of dimethylsulphoxide (DMSO) and if necessary heated to dissolve, 150μl of sesame oil (Invitrogen, The Netherlands) was then added and the mixture vortexed well. Control animals were injected with 200μl of control solution (50μl of DMSO and 150μl of sesame oil). 24hours later DRG were removed and fixed for 1hour on ice in 4% PFA in 1XPBS. The DRG were then washed 3 times with 1XPBS for 15mins each wash. The DRG were then placed in 100μl of DRG X-Gal solution (5mM K$_3$Fe(CN)$_6$, 5mM K$_4$Fe(CN)$_6$.6H2O, 1mM MgCl$_2$, 0.02% sodium deoxycholate, 0.02% NP-40, and 40mg/ml X-Gal [dissolved in DMSO] in 1 x PBS) and incubated at 37°C overnight. The X-Gal solution was then removed and the DRG placed in 70% v/v glycerol and stored at 4°C prior to photography.

6.1.2. FACS Analysis of Transfected Cells.
Transfected cells were washed x2 in 1XPBS and harvested by scraping with a 1ml-syringe barrel. Cells were resuspended in 500μl of 1XPBS and FACS analysis was performed directly. FACS analysis was performed by the department of immunology, UCL, London.

6.2. RESULTS.

6.2.1. Ecdysone Inducible Transgene Expression.
Results obtained previously indicated that the ecdysone regulatable gene expression system was promising in the context of a HSV-1 vector. Here replication incompetent ecdysone inducible vectors were created that contained both the transactivator and response elements. Based on previous results it was decided to construct a vector that contained the transactivator and response element in different regions of the HSV-1 genome. It was
hypothesised that this strategy would lower basal activity when compared to using a single expression cassette as seen with vector 1764pR20.8lacZ/UL43. This strategy was similar to that followed by others (Oligino et al. 1998) who created a mifepristone inducible HSV-1 expression vector.

6.2.1.1. Vector Design.

It was decided to separate the transactivator and response element of the ecdysone system within the HSV-1 genome. This would allow each part of the system to be studied in isolation and allow optimisation of both elements separately. To give maximal separation of the two elements, insertion into a UL gene and a US gene was thought reasonable. Previous work suggested that the UL43 and US5 genes would be suitable loci to use (see section 3.2.3.4.). Here, replication competent vectors with insertional inactivations in these genes were found to be capable of gene delivery to peripheral ganglia and in addition gave long-term transgene expression. US5 is a glycoprotein (gJ) (Ghiasi et al. 1998). US5 viral mutants have been shown to have normal phenotypes both in vitro and in vivo (Rasty et al. 1997; Weber et al. 1987) (Balan et al. 1994). UL43 is predicted to be a membrane channel protein but as yet has no known function (Carter et al. 1996). UL43 mutants have been shown to have a normal phenotype both in vitro and in vivo when compared to the parental virus strain (MacLean et al. 1991b). See section 3.2.3.1 for details.

Thus it was decided to clone a transactivator expression cassette into the US5 gene and the response element cassette into the UL43 gene. Two vectors were constructed, one of the vectors contained a LATP2 fragment (HSV nt 118866-120219) upstream to the ecdysone response element, the other was constructed without this fragment. The LATP2 promoter is known to drive low level latent transgene expression (Goins et al. 1994). In addition the promoter is known to confer a long-term enhancer effect on LAP1 and other promoters (Berthomme et al. 2000; Lokensgard et al. 1997) (Palmer et al. 2000) (see chapter 1). Here, it was speculated that the LATP2 element would enhance gene expression from the ecdysone response element in the long term, but would still allow regulated gene expression, as discussed previously.
6.2.1.2. Vector Production.

Details of the vector construction are shown below. Schematic diagrams are shown in figures 6.1 and 6.2.

1764 27-/P2-/4-/CMVGFP/US5

1764 27-/P2-/4- viral DNA had been produced previously (Lilley et al. 2001). The vector was based on the 1764 backbone deleted for ICP34.5/OrfP and inactivated for VP16, as described earlier. The vector was deleted for the ICP27 gene (HSV nt 113272-116869, Mlu to Mlu [this also deleted the nonessential genes UL55 and UL56]) and deleted in both copies of the ICP4 gene (HSV nt 126774 to 131730 (IRS) Sau3AI- Sphl) (Lilley et al. 2001). The vector was also deleted for the endogenous LATP2 regions (HSV nt 118768 to 120460). This DNA was transfected with pAT/5.1CMVGFP (Jill Smith, UCL, London) which had been linearised with XmnI, following protocol 2.2.6. The plasmid pAT/5.1 was pAT 153 (Invitrogen) missing a SamHI-EcoNI fragment (nt 375-626) and containing a BamHI–EcoNI HSV-1 insert encoding US5 (HSV-1 nt 136289-139328). The HSV insert had a SacI linker inserted within the US5 gene (HSV nt 137945). A CMV/GFP reporter cassette had been inserted in a unique BglII site within the SacI linker. Recombinant plaques were green when observed at 520nm and were purified away from white backbone plaques. The resulting vector was called 1764 27-/P2-/4-/CMVGFP/US5

pVIR/US5.

A MluI-Nsil/T4 fragment from pcDNA3VIR (laboratory plasmid, UCL, London) containing -CMV-VgEcR-IRES-RXR- was ligated into a EcoRI-BstXI/T4 fragment of pPIG19L (laboratory plasmid, UCL, London). This produced pPIL19VIR. Digestion of pPIL19VIR with Srl/PstI/Nsil/T4 gave a -CMV-VgEcR-IRES-RXR-pA fragment. This was ligated into pAT5.1 digested with Nsil/T4 (in the SacI linker). The fragment was cloned into the US5 locus in the forward direction.
1764 27-/P2-/4-/VIR/US5

1764 27-/P2-/4-/CMV/GFP/US5 viral DNA was transfected with pVIR/US5 (linearised with Pst1) following protocol 2.2.6. Recombinant plaques were white and were purified away from the green backbone. The resulting vector was called 1764 27-/P2-/4-/VIR/US5.

pE/GREZ-F/UL43.

pINDlacZ (Invitrogen) was digested with BglII/Sphl/T4 to release fragment -5xE/GRE-PΔHSP-LacZ-pA-. This fragment was inserted into p35mod (Suzanne Thomas, UCL London) which had been digested with NsiI/T4. p35mod is plasmid pGem2 (Invitrogen) containing UL43 flanking regions (HSV nt 91610-96751). The NsiI restriction site is within the UL43 locus (HSV nt 94911). Plasmids pE/GREZ-F and pE/GREZ-R were created that contained the -5xE/GRE-PΔHSP-LacZ-pA- fragment in the forward and reverse orientations respectively in relation to UL43 flanking regions.

pP2GZ-F/UL43.

pINDL19L (laboratory plasmid, UCL, London) was digested with SpeI/SrfI/Scal/T4 to release fragment -LATP2-5xE/GRE-LacZ-pA-. This fragment was ligated into p35mod digested with NsiI/T4 as previous. Two plasmids were produced pP2GZ-F and pP2GZ-R that contained -LATP2-5xE/GRE-LacZ-pA- in the forward and reverse orientations respectively in relation to UL43 flanking regions. The LATP2 fragment was HSV-1 nt 118 866 to 120 219.

1764 27-/P2-/4-/VIR/US5 P2GZ-F/UL43.

1764 27-/P2-/4-/VIR/US5 viral DNA was transfected with plasmid pP2GZ-F/UL43 (linearised with Scal) following protocol 2.2.6. Recombinant plaques were identified by incubation of virus with 10μg/ml ponasteroneA and X-Gal staining. Recombinant blue plaques were purified away from white backbone vector to produce vector 1764 27-/P2-/4-/VIR/US5 P2GZ-F/UL43.
1764 27/-P2/-4- /VIR/US5 E/GREZ-F/UL43.

1764 27/-P2/-4- /VIR/US5 viral DNA was transfected with plasmid pE/GREZ-F/UL43 (linearised with Scal) following protocol 2.2.6. Recombinant plaques were identified by incubation of virus with 10μg/ml ponasteroneA and X-Gal staining. Recombinant blue plaques were purified away from white backbone vector to produce vector 1764 27/-P2/-4- /VIR/US5 E/GREZ-F/UL43.
Figure 6.1. Schematic diagram to show production of ecdysone inducible replication incompetent vectors. Viral vectors are shown in back text boxes, plasmids are shown in red text boxes. Arrows represent transfections to produce new vectors. Plasmid VIR/US5 contains the ecdysone transactivator (VIR, [VgEcR-IRES-RXR]) in the US5 locus. Plasmid pP2GZ-F/UL43 contains LATP2, the ecdysone response element and the lacZ gene in the UL43 locus. Plasmid pE/GRE-Z/UL43 contains the ecdysone response element and the lacZ gene in the UL43 locus. Vectors A and B are identical other than that vector B contains LATP2 upstream to the ecdysone response element in the UL43 locus. Vector 1764 27-/P2-/4- is deficient in ICP34.5, VP16, LATP2, ICP27 and ICP4. See section 6.1.2.2. and figure 6.2 for details.
Figure 6.2. Replication incompetent ecdysone inducible vectors. Vector A 1764 27-/P2-/4- /VIR/US5 E/GREZ-F/UL43. Vector B. 1764 27-/P2-/4- /VIR/US5 P2GZ-F/UL43. Both vectors contain the ecdysone response element inserted into US5 in the forward direction. VgEcR, VP16/ecdysone receptor fusion. RXR, Retinoid-X-Receptor. VgEcR and RXR were cloned from pVgRXR (Invitrogen, The Netherlands) Nucleotide numbers refer to this plasmid. IRES, Internal ribosome entry site (pCITE; Professor J Almond, University of Reading). Vector A contains the ecdysone response element inserted into UL43 in the forward direction. Vector B contains LATP2 (HSV nt 118866-120219) upstream to the ecdysone response element inserted into UL43 in the forward direction. 5xE/GRE, Ecdysone/Glucocorticoid response element. P_{\text{HSP}}, heat shock minimal promoter. 5xE/GRE and P_{\text{HSP}} were cloned from VgRXR and nucleotide numbers refer to this plasmid.
6.2.1.3. In Vitro Characterisation of Plasmids.

Plasmids pVIR/US5, pP2GZ-F/UL43 and pE/GREZ-F/UL43 were verified using restriction digest analysis. Before vectors were constructed double transient transfection analysis of each plasmid together with the complementing transactivator or response element plasmid pIND/acZ or pVgRXR (Invitrogen) was performed. All plasmids were transfected at a ratio of 1:1. Transfection of pIND/acZ and pVgRXR was also performed. See table 6.1. Transfections were performed in duplicate, either with or without the addition of 10μg/ml of ponasteroneA (see protocol 5.1.1). These transfections were then repeated either 3 or 4 times. To determine β-galactosidase activity one set was fixed and X-Gal stained (protocol 2.2.4.3.) and the other 2 or 3 sets used in a luminometry assay (see protocol 2.2.4.2). Results can be seen in figures 6.3A and 6.3B. Figure 6.3B shows the fold induction of β-galactosidase activity upon addition of ponasteroneA. In each case figures are shown as an average of each set of transfections.

<table>
<thead>
<tr>
<th>Transactivator plasmid</th>
<th>Response element plasmid</th>
<th>Transfection ratio</th>
<th>Average fold-Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVgRXR</td>
<td>pINDIacZ</td>
<td>1:1</td>
<td>-</td>
</tr>
<tr>
<td>pVgRXR</td>
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<tr>
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<td>pE/GREZ-F/UL43</td>
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<tr>
<td>pVIR/US5</td>
<td>pINDIacZ</td>
<td>1:1</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Table 6.1. Details of transient tranfection assays.
Plasmids shown in bold italics were constructed for this thesis. The remaining plasmids are commercially available (Invitrogen).

Figures 6.3A and 6.3B clearly demonstrate that each of the plasmids constructed, pP2GZ-F/UL43, pE/GREZ-F/UL43 and pVIR/US5 are functional. In each case transient transfection with the complementary plasmids pVgRXR and pINDIacZ (Invitrogen) showed ligand inducible (ponA) β-galactosidase expression. The fold induction observed was variable within and between each set of transfections since in each case analysis relies upon both plasmids entering the same cell. Basal expression from the plasmids containing β-
galactosidase in the absence of ponA may also differ since each plasmid was different.
Figure 6.3A. Inducible transactivation of β-galactosidase after transient transfection of ligand inducible expression cassettes.

BHK cells were transiently transfected with transactivator and response element plasmids at a ratio of 1:1. 4 different transfections were performed, A-D.

A. pVgRXR + pINDlacZ  
B. pVgRXR + pE/GREZ-F/UL43  
C. pVgRXR + pP2GZ-F/UL43  
D. pVIR/US5 + pINDlacZ

Transfections were performed in duplicate. 24 hours post transfection new media was added either with or without 10μg/ml ponasterone A. 48 hours post transfection cells were fixed and X-Gal stained to determine β-galactosidase activity.
Figure 6.3B Inducible transactivation of β-galactosidase after transient transfection of ligand inducible expression cassettes.

BHK cells were transfected with transactivator and response element plasmids at a ratio of 1:1. Three different sets of transfections were performed A-C

A. pVgRXR + pE/GREZ-F/UL43
B. pVgRXR + pP2GZ-F/UL43
C. pVIR/US5 + pIND/acZ

Transfections were performed in duplicate, to be either incubated with or without ponasteroneA. 24 hours post transfection new media was added either with or without 10μg/ml ponasterone A. 48 hours post transfection cells were harvested and assayed for β-galactosidase activity. These transfections were repeated three times for A and B and twice for C. Fold induction shows the average within each set of transfections. Enzyme activity was measured using a Galacto-Light™ β-galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA) following protocol 2.2.4.2.
Vector 1764 27-/P2-/4- /VIR/US5 was constructed following figure 6.1. 7 different vector isolates were purified and a small stock of each was grown following protocol 2.2.6.2. Western blot analysis was performed in order to identify isolates that expressed the RXRa protein. 27/12/M:4 cells were infected at an MOI of 1 and cells harvested 24 hours post infection. Samples were prepared following protocol 2.2.7.2. Samples were run on a 10% acrylamide/10%SDS gel containing 2M urea. Previously a replication competent vector, 1764 pR20.8/lacZ/UL43, was shown to positively express the RXRa protein (see figure 5.6.). This vector was used as a positive control. Vector 1764 27-/P2-/4- /CMVGFP/US5 was used as a negative control (see section 6.1.2.1.). An RXRa antibody was used (Santa-Cruz Biotechnology). Results can be seen in figure 6.4. The RXRa protein is detected in the positive control and 6 out of 7 plaque isolates (1,3,4,5,6,7). Isolate #1 was chosen for further procedures since it appeared to produce a high amount of the RXRa protein.
Figure 6.4. Western blot detection of replication incompetent vectors expressing retinoid-X-receptor (RXRα). 27/12/M:4 or BHK cells were grown to 90% confluency and were infected with virus at an MOI 1 or 0.1 respectively. 24 hours post infection cells were harvested and samples prepared. Approximately 20µl of each sample were run on a 10% acrylamide/10% SDS gel containing 2M urea, alongside 5µl of molecular weight markers. A. 1764 27-/P2-/4- CMV/GFP/US5, negative control vector not expressing RXRα (see section 6.1.2.1.). B. 7 different isolates (1-7) of an ecdysone inducible vector 1764 27-/P2-/4- VIR/US5 (see section 6.2.2). C. 1764 pR20.8lacZ/UL43, positive control vector known to express RXRα (see figure 5.6). Detection with a monoclonal anti-mouse RXRα antibody (Santa-Cruz Biotechnology) diluted 1:500, reveals a 56kDa protein in the positive control 1764 pR20.8/lacZ/UL43 and 6 out of 7 (1,3,4,5,6 and 7) replication incompetent vectors isolates, 1764 27-/P2-/4- VIR/US5.
6.2.1.5. In Vitro Characterisation of Replication Incompetent Ecdysone Inducible Viral Vectors.

After construction of the two replication incompetent vectors, A. 1764 27-IP2-14-/VIR/US5 E/GREZ-F/UL43, B. 1764 27-IP2-14-/VIR/US5 P2GZ-F/UL43, which contained both the VgRXR genes and the inducible reporter gene cassette with or without LATP2, the vectors were tested in vitro. This served to check that the vectors were constructed correctly. During purification of the two vectors A and B, blue plaques were purified away from white backbone plaques of 1764 27-IP2-14-/VIR/US5 (see figures 6.1 and 6.2). During this procedure it became apparent that on 27/12/M:4 cells both vectors expressed lacZ in the absence of the activator ponA, although this activity did increase upon addition of 10μg/ml ponA (see figure 6.5). Thus, when replicating on complementing cells, the vector produced noticeable basal β-galactosidase activity. This basal activity was probably a result of transactivation of the ecdysone response element (5xE/GRE) and PΔHSP minimal promoter by viral proteins, as discussed previously (see section 5.2.10).

Following infection of non-complementing BHK cells basal β-galactosidase activity was reduced. BHK cells were infected in duplicate with vectors A or B at either an MOI of 5 or 10. These infections were then repeated. 24 hours post infection media was removed and new media added that was either with or without 10μg/ml ponA. 48 hours postinfection, lacZ activity was determined either by fixation of the cells and X-Gal staining (see protocol 2.2.4.3) or by luminometry assay (see protocol 2.2.4.2.). Results can be seen in figures 6.6A and 6.6B respectively. Figure 6.6A shows results of infection at an MOI of 5.

Figure 6.6A indicates that vector B has a high basal activity (-PonA) in comparison to that seen in figure 6.6B. However, when comparing the figures only the results at an moi of 5 in figure 6.6B are comparable. In addition when high levels of β-galactosidase activity occur, it has been observed that X-gal staining is often lower than expected due to some apparent saturation affect. Hence in figure 6.6A, upon addition of ponA, the X-Gal staining does not appear as strong as indicated by figure 6.6B, due to this saturation affect.
Figure 6.5. Phenotype of replication incompetent ecdysone inducible vectors on complementing cells. 27/12/M:4 cells were infected in duplicate at an MOI of 1 with vectors A and B, A. 1764 27-/P2-/4- VIR/US5 E/GREZ-F/UL43 and B. 1764 27-/P2-/4- VIR/US5 P2GZ-F/UL43. 24hours post transfection media was removed and new media added that was either with or without 10μg/ml ponasteroneA. 48hours post infection cells were fixed and X-Gal stained to determine β-galactosidase activity.
Figure 6.6A. Phenotype of replication incompetent ecdysone inducible vectors on non-complementing cells. BHK cells were infected at an moi of 5 with vectors A and B, A. 1764 27/-P2/-I4- VIR/US5 E/GREZ-F/UL43 and B. 1764 27/-P2/-I4- VIR/US5 P2GZ-F/UL43 24hours post infection media was removed and new media added that was either with or without 10μg/ml ponasteroneA. 48hours post infection cells were fixed and X-Gal stained to determine β-galactosidase activity.
Figure 6.6B. *In vitro* characterisation of ligand inducible replication incompetent viral vectors. BHK cells were infected in duplicate with vectors A or B at an MOI of either 5 or 10.

A. 1764 27^-/P2^-/4^- VIR/US5 E/GREZ-F/UL43
B. 1764 27^-/P2^-/4^- VIR/US5 P2GZ-F/UL43

24 hours post infection media was removed and new media was added either with or without 10μg/ml of ponasteroneA. 48 hours post infection cells were harvested and β-galactosidase activity determined using a luminometry assay (see section 2.2.4.2.). Fold induction at each MOI is displayed on the graph. See also figure 6.6A
The results show that unlike the replication competent ecdysone inducible vector, 1764pR20.8lacZ/UL43, both of the replication incompetent vectors, A and B, are inducible on BHK cells. Vector A. (1764 27-/P2-/4- VIR/US5 E/GREZ-F/UL43) showed a 35.2fold and a 28.3fold induction after infection at MOI's of 5 and 10 respectively. Vector B. (1764 27-/P2-/4- VIR/US5 P2GZ-F/UL43) showed an induction of 58.4fold and 39.4 fold after infection at MOIs of 5 and 10 respectively. Vector B, which contains LATP2 upstream to the ecdysone response element consistently gave the highest fold induction upon addition of ponA. It is interesting to note that at lower MOI's both vectors gave higher induction levels. Figure 6.6A shows the results of X-Gal staining of Vectors A and B on BHK cells. The pictures appear to show that vector A, (1764 27-/P2-/4- VIR/US5 E/GREZ-F/UL43) gives higher induction levels. However this may be an artefact of infection efficiency. Although the vectors were infected at the same MOI, it appears that vector B, (1764 27-/P2-/4- VIR/US5 P2GZ-F/UL43) infected more cells, such that in the absence of ponA a higher basal activity is observed and the presence of ponA a correspondingly higher level of β-galactosidase activity is observed. Alternatively the LATP2 sequence may be giving higher basal activity, ponA also giving improved induction with LATP2 and hence higher induction values.

6.2.1.6. Ex Vivo Analysis of Replication Incompetent Ecdysone Inducible Viral Vectors.

After the results observed in vitro vectors, A. (1764 27-/P2-/4-VIR/US5 E/GREZ-F/UL43) and B. (1764 27-/P2-/I4-VIR/US5 P2GZ-F/UL43) were tested for efficacy ex vivo. Each vector was injected unilaterally into the sciatic nerve of 6 mice at titres of; vector A. 3x10^6pfu/ml and vector B. 5 x10^6pfu/ml (see protocol 2.2.8.2.). 2days post inoculation DRG were extracted and incubated for 24hours in FGM either with or without 20μg/ml ponA (see section 5.1.2.). DRG were then fixed and X-Gal stained to determine β-galactosidase activity. The results can be seen in figure 6.7.
Figure 6.7. *Ex vivo* characterisation of ecdysone inducible expression vectors. 2-5 μl of vectors A and B were injected unilaterally into the rear sciatic nerve of mice at titres of 3x10⁵ pfu/ml and 5x10⁶ pfu/ml respectively.

**A.**
1764 27-/P2-/4-
VIR/US5
E/GRE-F/UL43

**B.**
1764 27-/P2-/4-
VIR/US5
P2GZ-F/UL43

2 days post inoculation DRG were extracted and incubated for 24 hours in FGM either with or without 20μg/ml ponA. DRG were then fixed and X-Gal stained to determine β-galactosidase activity. Only DRG incubated with ponA are shown here. Yellow arrows indicate positive cells.
The results here show that following explant of DRG and X-Gal staining very few lacZ positive cells were detected from either vector, even from those DRG incubated with 20μg/ml ponA. Figure 6.8 shows ganglia that were incubated with ponA. Only DRG positive for lacZ activity are shown. DRG incubated without ponA are not shown but gave similar results.

These results would lead to a number of hypotheses. The vectors did not reach the DRG, the ponA did not reach the vectors, the vectors were not responsive to ligand, or the transactivator protein was not expressed. In light of the in vitro results it would seem unlikely that the vectors were unresponsive to ligand, although in vivo this possibility cannot be discounted. The bioavailability of ponA to the DRG did not appear to be a problem when the replication competent vector 1764 pR20.8lacZ/UL34 was tested ex vivo (see section 5.2.9). As such it would seem unlikely that that this would be problematic here.

Recently in the laboratory replication incompetent vectors have been extensively tested in the CNS. It has been found that without LAP1/LATP2 upstream to the promoter/reporter cassette, no detectable levels of transgene expression are observed at locations connected to the injection site (i.e. following retrograde transport), even in the short term. Conversely the presence of LATP1 and LATP2, as seen when cassettes are placed directly into the endogenous LAT region (pR19 cassettes), leads to high-level transgene expression both in the striatum and substantia nigra following retrograde transport (Lilley et al. 2001). This would indicate that the problems observed here are possibly due to the lack of expression of the transactivator protein, a direct result of the absence of LATP2 from the transactivator cassette inserted into US5 (see figure 6.2). Thus gene expression following retrograde transport would now, with hindsight, not be observed. Significantly however, if the vector has successfully reached the ganglia then the basal β-galactosidase activity from the response element is extremely low even though high titres of the vectors were used, (3x10^8 pfu/ml and 5x10^8 pfu/ml). In conclusion it would seem that at present no definite conclusions can be drawn from this section of work.
6.2.1.7. In Vivo Analysis of Replication Incompetent Ecdysone Inducible Viral Vectors.

A separate experiment was performed using only vector B, (1764 27-l4-VIR/US5 P2GZ-F/UL43). This vector was chosen since it exhibited a higher degree of inducibility in in vitro studies. The virus was injected unilaterally into the sciatic nerve of 4 mice at a titre of 5x10⁷pfu/ml. 2 days post inoculation 2mg of ponA were injected IP into the ipsilateral side of two mice. Two mice were used as controls (see section 6.1.1). 24 hours later DRG were extracted and X-Gal stained to determine β-galactosidase activity. Results are not shown but were very similar to the results obtained in the ex vivo experiments (see figure 6.7) where very few lacZ positive cells were observed. Again, possibly the vector or ponA did not reach the DRG. However it now seems more likely that this result can be explained by the lack of expression of the transactivator protein, due to the absence of LATP2 in the transactivator cassette, as discussed in the last section.

It is unknown whether ponA will reach the DRG following IP injection. Others have reported that following IP injection of 750μg of ponA the ligand reached the mammary glands of transgenic mice, with effective induction achieved at even lower doses (Albanese et al. 2000). In addition, following IP injection of 10mg of murA, the ligand was shown to reach the thymus of transgenic mice (No et al. 1996). Skin and tail biopsies of transgenic mice showed that following IP injection of 3-5 mg of ponA or murA that the ligands were capable of reaching these tissues (Saez et al. 2000). Here it was shown that reporter gene induction in the skin began to trail off around 1mg of ponA. Collectively these results suggest that the inducing ability of ponA may differ between tissues. Thus it may be necessary to administer higher doses of ponA in order for the ligand to mediate transactivation from a viral vector from within this tissue. This possibility remains to be tested.
6.2.2. Tetracycline Repressor Inducible Transgene Expression.

It was observed previously that a 'Tet-On' ligand regulatable expression cassette and corresponding HSV-1 vector performed poorly in terms of regulated gene expression, the highest induction observed being 4.2-fold. Thus it was decided to test the tet repressor system, an arrangement whereby the tetracycline repressor (tetR) alone, rather than tetR-mammalian cell transcription factor fusion derivatives (rTtA), can function as a potent transmodulator to regulate gene expression (Yao et al. 1998). For the purposes of the work described here, it was hoped that the binding of the tet-repressor to the tet-operator sites would minimise basal leakiness of the reporter genes. Either as a result of cellular transactivating factors, as observed in BHK cells (Ackland-Berglund and Leib 1995) or by HSV-1 proteins such as ICP4 and ICP0 (Herrlinger et al. 2000).

6.2.2.1. Vector Design.

It was decided to clone the elements of the tet-repressor system into a single regulatory cassette in order to compare the results directly with those obtained previously with the pR20.4/lacZ cassette (see figure 5.1). The two components of the system were the tet-repressor, TR (pcDNA6/TR; Invitrogen) and the promoter-tet-operator, TO (pcDNA4/TO; Invitrogen) See figure 1.12. Two different strategies were undertaken. Firstly, a bicistronic cassette was created such that the elements TO and TR were inserted in a back-to-back arrangement, transcription coming from two different promoters positioned in opposite directions, pR20.1/27/TO/TR. This was similar to the arrangement of the 'Tet-On' cassette, pR20.4/lacZ. Secondly, a cassette was designed to incorporate an autoregulatory loop, pR19TO/TR/IRESGFP. A schematic representation of both cassettes can be seen in figure 6.8.

It was expected that low level basal transcription would occur from the pR19TO/TR/IRESGFP cassette such that minimal levels of TO, TR and GFP produced would be expressed. However, as soon as the TR protein is assembled it will bind to the TO sequences and repress gene expression. Thus an autoregulatory loop is set up. The advantage of this system is that since expression of TO and TR comes from the same promoter, (Pcmv), expression is...
not dependent upon the regulation and activities of two different promoters possibly with different levels of activity. A retrovirus containing a 'Tet-Off' autoregulatory cassette has been described previously (Hofmann et al. 1996). Here the tet-operator sequences (tetOx7CMVTATA) were cloned upstream to the lacZ gene. Downstream to lacZ was an IRES followed by the tetracycline controlled transcriptional transactivator, tTA. Minimal transcription from the tet operator sequences allowed basal expression of lacZ and tTA. In the absence of tet the tTA binds to the tetO sequences in an autoregulatory loop and causes up-regulation of lacZ expression. In the presence of Tet, the tTA cannot bind to the tetO sequences and only basal lacZ expression is observed. The group found that upon retroviral integration into primary myoblast cells induction of one or two orders of magnitude were observed (Hofmann et al. 1996). The autoregulatory loop described by Hofmann et al is one of positive feedback such that in the absence of tetracycline gene expression occurs. However in the pR19/TO/TR/IRESGFP cassette the autoregulatory loop is one of negative feedback such that in the absence of tet transcription is repressed.

Using the pR20.1/27/TO/TR and pR19/TO/TR/IRESGFP cassettes it was decided to construct replication incompetent tet-repressor vectors, deleted for ICP34.5/OrfP, ICP4, ICP27 and deficient in VP16. The pR19/TO/TR/IRESGFP cassette would be inserted in the LAT region similarly to vectors described previously (see figure 4.4). The pR20.1/27/TO/TR cassette would be inserted into ICP27 flanking regions. These flanking regions were constructed such that upon recombination into the vector backbone they would remove endogenous LAP1 and LATP2 regions in the internal long repeat region, IRL. This was necessary in order to prevent homologous recombination between the endogenous LAT region and the LAT region in the inserted cassette. This had previously been found to be a problem when making insertions into ICP27 since the gene is relatively close to the LAT region in the HSV-1 genome (Caroline Lilley, personal communication).
6.2.2.2. Plasmid Construction.
Plasmid construction is detailed here, each plasmid required a three step cloning strategy. A schematic diagram of resulting cassettes can be seen in figure 6.8.

pR20.1/27/TO/TR

pR20.1/27LAT

pΔ27Lat is pSP72 (Promega) containing HSV ICP27 flanking regions. These flanking regions were designed to result in the deletion of the region between HSV nucleotide 113273 (MluI) and nucleotide 120300 (Hpal). This would result in the deletion of ICP27, UL55, UL56, LAP1 and LATP2. The flanking regions were cloned into pSP72 such that they were separated by a BglII site. Plasmid pΔ27LAT was digested with BglII/T4. pLL19G (laboratory plasmid, UCL, London) was cut with SrfI to release fragment -pA-lacZ-LAP1-LATP2-CMV-GFT-pA-. The SRFI fragment was cloned into p27LAT at the BglII site to produce plasmid pR20.1/27LAT-F. The LATP2 fragment in the plasmid was in the same orientation (forward) to the endogenous LATP2.

pR20.1/27LAT/TO

pR20.1/27LAT was digested with SpeI/T4/HindIII to release CMV. PCDNA4/TO (Invitrogen) was digested with NruI/T4/HindIII to release a tet operator fragment, TO, (nt 208-978). The TO fragment was cloned in place of CMV in pR20.1/27LAT to produce pR20.1/27LAT/TO.

pR20.1/27/TO/TR

Plasmid pcDNA6/TR (Invitrogen) was digested with MspAI/T4/NheI to release fragment -globin IVS-TetR- (nt 895-2312), which was cloned into pR20.1/27LAT/TO missing an EcoRI/T4/XbaI fragment (-lacZ-). Plasmid pR20.1/27/TO/TR was produced. See figure 6.8.
**pR19TO/TR/IRESGFP**

**pcDNA4TO/TR**

pcDNA6 was digested with Nhel/T4/BstXI (nt 895-2491) to release a -globinIVS-TetR-SV40pA- fragment. This fragment was cloned in pcDNA4/TO invitrogen) digested with HindIII/T4/BstXI (nt 978-1041). Plasmid pcDNA4/TO/TR was produced.

**pR19TO/TR/IRES/GFP(spe-)**

pcDNA4TO/TR was digested with MspAlII/T4/SpeI to release fragment -PCMV-2xTetO2-globinIVS-TetR- (TO/TR). This fragment was cloned into pR19IRESGFP (laboratory plasmid, UCL, London) missing an EcoRI/T4/SpeI (-LAP1-LATP2-CMV-) fragment. This produced plasmid p19TO/TR/IRESGFP(spe-).

**pR19TO/TR/IRESGFP**

pR19TO/TR/IRESGFP(spe-) was linearised with SpeI. A 1.7kb SpeI fragment from pR19IRESGFP was Inserted into this SpeI site (-LAP1-LATP2- [LAP1-HSV nt 118443-118878] [LATP2 HSV nt 118866-120219]). This produced pR19TO/TR/IRESGFP. See figure 6.8.
Figure 6.8. Schematic representation of ligand inducible expression cassettes containing the tet-repressor system.

A. pR20.1/27/TO/TR and B. pR19TO/TR/IRESGFP

LAP1 and LATP2, HSV-1 latency associated promoters. TetR and globin IVS, Tetracycline repressor and rabbit β-globin intron II (nucleotide numbers refer to pCDNA6/TR [Invitrogen, the Netherlands]). PCMV 2xTetO2, Cytomegalovirus promoter and Tet operator (x2) sequences (nucleotide numbers refer to pcDNA4/TO [Invitrogen, The Netherlands]). IRES, internal ribosome entry site. GFP, green fluorescent protein. pA polyadenylation signal. Arrows represent direction of promoters or genes.
6.2.2.3. **In vitro Characterisation of Tet-Repessor Plasmids.**

The tet represor plasmids, pR20.1/27/TO/TR and pR19/TO/TR/IRESGFP were characterised *in vitro* for tetracycline regulated gene regulation. BHK cells were transiently transfected in duplicate with 5μg of plasmid. 24hours post transfection new media was added that was either with or without 1μg/ml of tetracycline. 48hours post transfection cells were observed under UV light at a wavelength of 520nm. Results can be seen in figure 6.9. This experiment was then repeated twice using either 1μg or 5μg of plasmid and this time after 48hours cells were harvested and analysed by FACS analysis (see section 6.1.2). Results are shown in table 6.2. Mock transfected BHKs were used as the control population of cells allowing gating (M1) for analysis of fluorescent intensity in the FL-1 green channel at 520nm.

<table>
<thead>
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<th>Plasmid</th>
<th>% of cells within M1 +Tetracycline</th>
<th>% of cells within M1 -Tetracycline</th>
<th>Fold Induction</th>
</tr>
</thead>
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<tr>
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<td></td>
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<td>1μg</td>
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</tr>
<tr>
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<td>9.39</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 6.2. FACS analysis of tet-repressor constructs.** BHK cells were transiently transfected in duplicate with plasmid constructs in the presence or absence of tetracycline. 48hours post transfection cells were harvested and FACS analysis was performed. Figures refer to the % of cells within the live-gate that fell within marker M1 and thus showed a shift in fluorescent intensity compared to mock transfected cells.

Results show that neither plasmid pR20.1/27/TO/TR or plasmid pR19/TO/TR/IRESGFP appeared to be responsive to tetracycline regulated gene expression. Following transient transfection and observation at 520nm (figure 6.9) high levels of basal GFP expression were observed from the pR20.1/27/TO/TR plasmid in the absence of tet that matched those seen in the presence of tet. Low level GFP expression was observed from the
pR19TO/TR/IRESGFP plasmids both in the presence and absence of tetracycline. The lower levels of GFP expression seen with the pR19TO/TR/IRESGFP plasmid, compared to the pR20.1/27/TO/TR construct, were expected due to the positioning of the GFP gene downstream to an IRES and after both TO and TR genes. Upon closer examination of similarly transfected cells using FACS analysis, the observed results were confirmed and showed that for both plasmids tet regulated gene expression was not detected. No conclusions could be drawn from the very small fold induction observed.
Figure 6.9. *In vitro* characterisation of inducible expression cassettes containing the tet-repressor system. BHK cells were transiently transfected in duplicate with plasmids A and B. 

**A.** pR20.1/27/TO/TR  

**B.** pR19TO/TR/IRESGFP  

24 hours post transfection new media was added either with or without 1µg/ml tetracycline. 48 hours post transfection cells were observed under a fluorescent microscope at a wavelength of 520nm.
6.2.2.4. **In Vitro Characterisation of Tet-Representer Plasmids on T-Rex™-HeLa Cells.**

It had been shown previously that plasmids pR20.1/27/TO/TR and pR19/TO/TR/IRESGFP were unresponsive to tetracycline regulated gene expression on BHK cells. Since this result could be related to the cell line used it was decided to test the cells on T-REx™-HeLa cells (Invitrogen). This cell line constitutively expresses the tetracycline repressor (TR) from pcDNA6TR (Invitrogen). It is known that the efficacy of the 'Tet-Off' tetracycline regulated system is influenced by cell type (Ackland-Berglund and Leib 1995). The original 'Tet-Off' system was created using HeLa cells (Gossen and Bujard 1992) and the tet-repressor system was shown to be efficient after transient transfection in HeLa cells (Yao *et al.* 1998). Thus, it was speculated that if the plasmids failed to show tet regulated gene expression on HeLa cells then they were likely to be incorrect. Restriction digest analysis had verified the constructs but this process would not detect any small mutations/errors that could have occurred in construction.

5μg of plasmids pR20.1/27/TO/TR and pR19/TO/TR/IRESGFP were transiently transfected onto T-REx™-HeLa cells in duplicate following protocol 2.2.4.1. 24 hours post transfection new media was added either with or without 1μg/ml tetracycline. 48 hours post transfection cells were observed under UV light at a wavelength of 520nm. Results of the pR20.1/27/TO/TR plasmid can be seen in Figure 6.10.

Figure 6.10. shows that in T-REx™-HeLa cells the pR20.1/27/TO/TR plasmid is responsive to tetracycline. In the absence of tetracycline and thus when the tet repressor is bound to the tet-operator sites, no GFP expression was detected. However, in the presence of 1μg/ml tetracycline high levels of GFP expression were observed. These results indicate that BHK cells may not be a suitable cell line in which to test tet-repressor plasmids, as reported previously for the 'Tet-Off' system (Ackland-Berglund and Leib 1995). Results from the pR19TO/TR/IRESGFP are not shown, however in both the presence and absence of tetracycline no GFP expression was detected.
Due to time constraints it was not possible to test the pR20.1/27/TO/TR plasmid on other cell lines such as ND7 cells or HeLa cells not expressing the TR plasmid, or to construct a vector expressing pR20.1/27//TO/TR.
6.10. *In vitro* characterisation of pR20.1/27/TO/TR on a T-REx™-HeLa cell line expressing the tet-repressor.

T-REx™-HeLa cells (Invitrogen) were transiently transfected in duplicate with plasmid pR20.1/27/TO/TR, which contained the tet-repressor regulatable system. 24 hours post transfection new media was added either with or without 1 μg/ml tetracycline. 48 hours post transfection cells were observed under a fluorescent microscope at a wavelength of 520 nm.
6.3. DISCUSSION.

The aim of this section of work was to construct a ligand regulatable replication incompetent HSV-1 vector. It has been shown previously by us and others that HSV-1 proteins interact with and deregulate the ecdysone and tetracycline inducible expression systems, see chapter 5 (Herrlinger et al. 2000). Thus in an attempt to create a successful regulatable HSV-1 vector it was logical to use a vector backbone incapable of expressing any proteins linked with transactivation of inducible promoters, such as ICP4, ICP0, ICP27 and VP16. Such a vector had previously been created in the laboratory (Lilley et al. 2001). On non-complementing cells the only viral protein produced at significant levels by this vector is ICP6 (Lilley et al. 2001). Work here describes the production of two ecdysone regulatable replication incompetent vectors and their characterisation both in vitro and in vivo. In addition the construction of two tet-repressor regulatable plasmid constructs is described and their characterisation in vitro.

The ecdysone inducible expression system was developed from the ecdysone receptor of Drosophila melanogaster (DmEcR)(No et al. 1996). As yet no viral vectors have been described that use this regulatable system. The ecdysone inducible replication incompetent vectors described here were similar to one another apart from vector B (1764 27-/P2-/4-VIR/US5 P2GZ-F/UL43), had LATP2 (HSV nt 118866-120219) inserted upstream to the ecdysone response element and vector A, (1764 27-/P2-/4-VIR/US5 E/GREZ-F/UL43) did not. LATP2 is a downstream extension of LAP2 (Goins et al. 1994) and was identified in our lab (Palmer et al. 2000). Part of the design strategy was to assess the effects of LATP2 on regulatable gene expression.

*In vitro* transient transfection analysis of the plasmids used to construct the vectors, pVIR/US5, pE/GRE-F/UL43, pP2GZ-F/UL43 with the corresponding plamids pVgRXR and pINDiacZ, verified that each was correct and mediated ponA induced β-galactosidase expression (see figures 6.3A and 6.3B). The fold induction observed was not comparable between each set of transfections since the results were dependent upon how many cells in each case were co-transfected with both plasmids and the basal expression of each of the different
β-galactosidase expressing plasmids. It would have been sensible to perform transfections of the response element plasmids alone, in the absence of transactivator or effector, to determine basal expression levels. However since the objective of the experiment was to determine whether the plasmids were functional and not the absolute levels of regulation, this was not undertaken.

The efficacy of the ecdysone vectors A and B(+P2) was tested both in vitro and in vivo. In vitro analysis was performed in BHK cells and showed a 32.2fold and 28.2fold induction for vector A and a 58.4 and 39.4fold induction for vector B(+P2). See figure 6.6A and 6.6B. Although vector B showed the greater induction of the two vectors it also appeared to show the greater basal activity (see figure 6.6A) such that even in the uninduced state (-ponA) high levels of β-galactosidase activity were observed. The basal activity in both cases could be explained by the presence of residual levels of HSV-1 proteins such as ICP4, ICP0, ICP27 and VP16, in the stock of vectors used. In addition the higher basal activity observed from the LATP2 containing vector could be as a result of up-regulation of lacZ expression directed by the LATP2 element itself or conferred upon the hCMV promoter by LATP2. It has been suggested previously that ICP4 or ICP0 may function to repress the activity of LAP2 (Goins et al. 1994) and it is known that there is an ICP4 binding motif in the LAP1 promoter that down-regulates the promoter during productive infection (Batchelor et al. 1994). Thus it would seem unlikely that the presence of LAPT2 would cause an upregulation in ligand inducible gene expression but the possibility of this should not be discounted.

The ecdysone inducible vectors described here contained the transactivator and response element inserted into separate regions of the HSV genome. It would have been interesting to compare these replication incompetent vectors with a replication competent vector containing the single expression cassette pR20.8lacZ/UL43, described in chapter 5. This could be done in the future.

One of the problems with the ecdysone inducible expression system is the cost of the effectors ponasteroneA and muristeroneA. The cost was such that it was limiting for the purposes of this thesis and meant that extensive in vivo
characterisation of the ecdysone inducible vectors described here was not feasible (as discussed in chapter 5). Other groups have utilised a modified form of the ecdysone inducible system (Hoppe et al. 2000) that uses an ecdysone receptor from Bombyx mori (BmEcR). This receptor is capable of full transactivation without the need for exogenous RXR and in addition has an enhanced induction compared to the Drosophila melanogaster EcR (DmEcR) by particularly potent non-steroidal ecdysone analogues such as GS-E, (N-[3-methoxy-2-ethylbenzoyl]-N'-[3,5-dimethylbenzoyl]-N'-tert-butylhydrazine) (Hoppe et al. 2000). Thus, as an alternative route it might be possible to utilise this system in terms of a HSV-1 vector and thus have the facility for full in vivo analysis.

The efficacy of the ecdysone inducible vectors was tested ex vivo. Results here were inconclusive since very few lacZ positive cells could be identified in DRG after injection of either of the vectors and incubation in the presence or absence of ponA. However it is speculated that the reason for this is the lack of, or very low levels of, transactivator protein. This could possibly be due to the absence of the LATP2 promoter in the US5 transactivator cassette. It would be sensible to perform in situ hybridisation, RT-PCR or northern blot analysis in order to detect the presence or absence of HSV LATs and thus determine if the vectors are actually reaching the ganglia. In addition immunohistochemistry could be performed on sections of DRG in order to test for expression of RXR form the transactivator. Considering the expense of the effector ponA it would also be sensible to do this before undertaking any further in vivo analysis. If it is concluded that the vector can reach the DRG then future work might include the creation of a similar replication incompetent vector but with the transactivator inserted directly into the endogenous LAT region, instead of US5, in order to utilise the LAP1 and LATP2 promoters. In addition to this it might be sensible to assess the extent of delivery of ponA to the DRG tissue following IP injection. This has been done previously in mammary gland tissue (Albanese et al. 2000). HPLC purification of ponA within mammary gland tissue was performed and the activity assessed using a plasmid based reporter assay.

The tet repressor tetracycline regulated system has been described previously (Yao et al. 1998). Work in this section describes two plasmid constructs,
pR20.1/27/TO/TR and pR19/TO/TR/IRESGFP, each of which contains both elements of this system the tet-repressor (TR) and tet-operator (TO). In vitro analysis of these expression cassettes revealed that only pR20.1/27/TO/TR construct appeared to be regulatable and then only in a cell line (T-REX™-HeLa) that expressed the tet repressor. It has been proposed that when using the tet-repressor system that the tet-repressor and tet-operator elements should be used at a ratio of 6:1 for efficient repression to take place (Yao et al. 1998). Since a ratio of 6:1 was not used in the systems described here, this could explain the lack of regulation observed. However, the ratio refers to optimal transgene repression and even at lower ratios repression should still be detected (Yao et al. 1998).

Since the cassettes described here were to be used in the context of viral vectors results here highlight the advantages of using the two vector approach used by others (Harding et al. 1998) (Rendahl et al. 1998). Here it is possible to use the vectors at different ratios to achieve maximal gene regulation. Harding et al found that a ratio of 1:20 transactivator to response element was necessary using adenoviral vectors in the rat hippocampus. In contrast to this Rendahl et al used a ratio of 10:1 transactivator to response element when using AAV vectors to regulate erythropoietin expression in the muscle of adult mice. Thus it would seem that the ratios used would need to be tailored to suit each vector system individually. However, both of these systems rely on the fact that cells will be transduced efficiently and consistently with both vectors. A distinct advantage of the one vector approach is that this will not be a problem.

As described previously a reason for the lack of transgene repression was possibly due to the lack of expression of the tetR protein. Western blot analysis of extracts of BHK cells transiently transfected with both the tet-repressor plasmids was performed in order to detect the tet repressor protein (results not shown). A rabbit anti-mouse TetR monoclonal antibody was used (BD Clontech, Basingstoke) see section 2.2.7.5. However, the procedure consistently failed to detect tetR even from extracts of T-REX™-HeLa cells known to express TetR, which were used as the positive control (see section 6.2.2.4). Further work should be performed in order to establish if the constructs actually do express
the tetR protein. Alternatives to western blot analysis would be northern blot analysis or sequencing of the construct.

The pR19TO/TR/IRESGFP cassette contained an autoregulatory loop to regulate GFP expression. However no evidence of this regulatable GFP expression was observed. Interestingly however the GFP expression detected was very low. This may prove beneficial since low basal gene expression is required in order for the autoregulatory loop to work. Thus the low basal activity will only result in limited GFP expression. In addition it may have been sensible to clone the TR gene upstream to TO such that this might encourage elevated expression of TR compared to TO. This would fit in with the 6:1 ratio of tet-repressor to tet-operator that is required for optimal gene repression (Yao et al. 1998).

In conclusion two ecdysone regulatable viral vectors have been described that mediate up to a 58.4fold induction of β-galactoidase activity in vitro on non-complemetning cells. In vitro and ex vivo work suggests that ecdysone inducible vectors will be functional in the PNS. Further work would sensibly involve the generation of a vector containing the ecdysone transactivator in the LAT region such that it is in close proximity to LATP2. In addition the bioavailability of ponA will need to be determined. Two tet-repressor plasmids have also been described, that show limited efficacy and which require further development and characterisation. The successful development of both of these systems will enable gene regulation in neuronal cells. This will facilitate the study of gene function, especially cytotoxic genes (Hermens and Verhaagen 1998). In addition it will be useful in many gene therapy protocols where continuous gene expression is not always required.
CHAPTER 7:

APPLICATIONS IN THE PNS
CHAPTER 7: APPLICATIONS IN THE PNS.

7.0. INTRODUCTION.
The development of successful gene therapy vectors provides a means to study the role of proteins in neurological disease. The preceding chapters have discussed the development of various disabled HSV-1 vectors and gene delivery to peripheral ganglia. The focus of this section of work describes the use of these vectors in two model systems, using the Cre/loxP system for conditional gene alteration and testing the neuropeptide galanin in relation to its potential nerve regeneration capacity.

7.0.1. Cre Recombinase.
Cre is a 38kDa recombinase protein from bacteriophage P1, that mediates recombinational events between two loxP sites (Sternberg and Hamilton 1981). LoxP sites are 34bp in size and consist of two 13bp inverted repeats, separated by a 8bp spacer, the spacer gives the site its orientation. The inverted repeats provide the binding sites for Cre such that two molecules bind per loxP site. Two loxP sequences in opposite orientations direct inversion of the intervening piece of DNA, two sites in the same orientation direct excision of the intervening DNA leaving one loxP site behind. LoxP sites on separate chromosomes can mediate translocation between the chromosomes (reviewed in van der Neut 1997).

The Cre/loxP system has been extensively studied in prokaryotic systems (Austin et al. 1981; Abremski and Hoess 1984; Hamilton and Abremski 1984). In the late 1980s the system was shown to be functional in eukaryotes allowing new approaches in gene targeting and transgenic technology (Sauer and Henderson 1988; Sauer and Henderson 1989; Sauer and Henderson 1990). Many approaches in gene targeting, such as knockout and transgenic mice, have meant that gene alterations are present in the germline and are thus manifest in all the cells at all times. Thus, if the genetic change has a lethal phenotype at early stages of development then later phenotypes cannot be assessed. In addition many genetic changes give rise to pleiotrophic effects
and such phenotypes could be more easily studied if alterations were restricted to one tissue or phenotype. The Cre/lox system provides a means by which spatial and temporally controlled genetic modifications can be addressed and is widely used in the generation of exon specific or cell type specific gene knockout in mice (van der Neut 1997). Exon specific gene knockouts provide a means by which the role of specific splice variants can be studied. A tissue specific knockout has been used to delete the DNA polymerase-β gene specifically in T cells, by virtue of Cre recombinase expression driven by a T-cell specific promoter (Gu et al. 1994).

There have been many reports detailing the use of viral vectors for the delivery of Cre recombinase to desired tissues or cells. Many of these reports utilise adeno-viral or retroviral vectors, either for the excision of floxed genes (genes flanked by loxP sites) from plasmids (Anton and Graham 1995 Sato et al. 1998), the excision of floxed genes from other viral genomes (Anton and Graham 1995; Fernex et al. 1997), or for excision from cell lines or specific cell populations (Piston et al. 1999; Stec et al. 1999). Stec et al (Stec et al. 1999) constructed an AdCre vector expressing Cre under the control of the HCMV promoter. The group was interested in examining the tissue specific loss of angiotensinogen (AGT) gene function in vivo. They created a transgenic mouse strain with a human angiotensinogen gene flanked by loxP sites (hAGT\textsuperscript{lox}). Circulating AGT is produced mainly in the liver and it is known that adeno-viruses delivered intravenously infect mainly the liver. Thus, here was a system whereby tissue specific delivery of a transgene could be achieved in a temporal fashion without the need for tissue specific or a regulatable promoter. The tissue specificity of the AdCre was validated by southern blot analysis. This group reported that after Ad administration in two mouse lines, circulating levels of hAGT (plasma) were reduced by 90-100%, clearly demonstrating that viral vectors are an effective tool for the knockout of floxed genes in transgenic animals.

The Cre/lox system has also been used for the construction of viral packaging cell lines (Parks et al. 1996; Vanin et al. 1997; Tashiro et al. 1999). Possibly the
most useful of these is a HEK293 cell line used in the generation of gutless adenoviral vectors, see figure 1.4 (Parks et al. 1996).

There have been a number of reports describing the use of Cre recombinase in HSV (Brooks et al. 1997; Rinaldi et al. 1999; Logvinoff and Epstein 2000). A number of amplicon or cosmid based systems have been described (Brooks et al. 1997; Logvinoff and Epstein 2000). Logvinoff et al reported that the Cre/loxP system was functional in the context of cells infected with HSV-1 and thus suggested that the technology could be used to engineer the genome of HSV-1 or HSV-1 based vectors in cultured cells. The authors were concerned here that expression of vhs from the vector, which induces mRNA degradation (Kwong et al. 1988) would lead to shut off of cellular protein synthesis. However this was found not to be the case. They created a cosmid based HSV-1 vector containing a floxed gene blocking expression of lacZ. They showed that upon infection of a human cell line constitutively expressing Cre recombinase, recombination between the loxP sites in the HSV genome allowed expression of β-galactosidase. Furthermore they showed that an amplicon carrying multiple loxP sites could undergo loxP recombination both in cells constitutively expressing Cre recombinase and in naive cells having received amplicons expressing Cre. Brooks et al (Brooks et al. 1997) constructed a HSV-1 amplicon vector to deliver Cre recombinase to the hippocampus of transgenic mice. The mice carried a nerve growth factor (NGF) excision-activation transgene that would only be expressed following excision of a floxed-inactivating cassette upstream to NGF. Following stereotactic delivery of the HSVCre vector to the mouse brain and unilateral expression of the NGF transgene as a result of Cre mediated excision, mice displayed an increase in locomoter activity compared to a control group. This was the first reported HSV-1 vector capable of making a conditional genomic alteration in mice.

Rinaldi et al (Rinaldi et al. 1999) created disabled HSV vectors mutated in VP16, ICP0 and ICP4 (Preston et al. 1998) that expressed either Cre recombinase or a lacZ gene, expression of lacZ was blocked by an upstream floxed antibiotic resistance gene. They showed that both vectors were functional in vitro, on Vero cell lines and in addition were functional in vivo.
analysis was performed by inoculation of both vectors into mouse footpad and extraction of DRG and X-Gal staining. Results showed that *lacZ* positive neurons were detected, the result of Cre recombinase activity within the vector genome.

It is anticipated that HSV-1 Cre recombinase expression vectors described in this thesis will be used to study the role of voltage gated sodium channels in pain perception. It is known that two tetrodotoxin sensitive voltage-gated sodium channels are preferentially expressed in small diameter sensory neurons of the PNS, which include nociceptive cells (Akopian *et al.* 1996; Sangameswaran *et al.* 1996; Dib-Hajj *et al.* 1998; Tate *et al.* 1998). These channels have been termed SNS (Akopian *et al.* 1996; Sangameswaran *et al.* 1996) and NaN (Dib-Hajj *et al.* 1998; Tate *et al.* 1998). Previously an SNS null mutant mouse facilitated the study of SNS channels and their role in pain perception, concluding that blockade of SNS expression may produce analgesia without side effects (Akopian *et al.* 1999). Since HSV-1 naturally infects sensory neurons in peripheral ganglia, it is anticipated that delivery of a vector expressing Cre recombinase, to peripheral nerves will result in the conditional knockout of the SNS sodium channel in transgenic mice. This will provide a quick and easy way to further characterise the role of SNS channels in pain perception. The use of a disabled HSV-1 vector will allow for Cre recombinase expression in a spatially restricted manner without the need for a tissue specific promoter.

**7.0.2. Galanin.**

Peripheral nerve damage causes changes within neuronal cell bodies that are thought to promote cell survival, axonal regeneration and functional recovery. One of the most potent changes in the DRG following peripheral nerve injury is the 120-fold increase in the levels of galanin (Hokfelt *et al.* 1987). Other changes include the down-regulation of substance P and calcitonin gene-related peptide (CGRP) and the up-regulation of vasoactive intestinal peptide (VIP) (Zigmond *et al.* 1996). Galanin is a neuropeptide of 29 amino acids (Tatemoto *et al.* 1983) that is expressed in high levels in most cells of the DRG from day 16 of gestation until shortly after birth (Xu *et al.* 1996). In adult DRG
galanin is expressed in only 2-3% of DRG cells which are predominantly small fibre neurons (Hokfelt et al. 1987). Following axotomy mRNA and peptide are abundantly expressed in 40-50% of all DRG neurons (Hokfelt et al. 1994) and remain elevated whilst the nerve is regenerating.

A transgenic mouse line has been generated that contains a loss of function mutation in the galanin gene. This knockout line has been used to demonstrate that the chronic absence of galanin throughout development causes attenuation in chronic neuropathic pain behaviour (Kerr et al. 2000). Most recently the same knockout line has been used to investigate the role of galanin in the somatosensory system after injury. Results suggested that small diameter peptidergic nociceptive neurons were preferentially lost in galanin knockout animals and in addition axonal outgrowth and regeneration are reduced in DRG sensory neurons of these animals (Holmes et al. 2000).

It was anticipated that a HSV-1 galanin expression vector would be used to rescue the in vivo and in vitro phenotype of the galanin knockout animal (Wynick et al. 1998). In vitro DRG cultures from galanin knockout animals show both a reduction in the number of cell producing neurites and a deficit in neurite length compared to wild-type animals (Holmes et al. 2000). In vivo and following sciatic nerve crush mutant animals show a reduction in the regeneration distance of the sciatic nerve compared to wild type animals, as measured by the pinch test, GAP43 visualisation of growth cones and behavioural toe spreading index analysis (Holmes et al. 2000).

7.0.3. Summary.

This section of work describes the construction of disabled viral vectors containing the genes for Cre recombinase and galanin. The vector genotypes verified were tested in vivo and the possible uses of such vectors have been suggested. Previous work in chapters 3 and 4 indicated that different vector backbones would be suitable for different biological and clinical applications. For example a replication incompetent vector, when used at high titre would be suitable for long-term non-toxic gene delivery at consistent levels (chapter 4). Conversely, a replication competent vector containing the pR20.5 expression
cassette would be suitable for high levels of gene delivery but only if such high levels are required in the short term (chapter 3). In addition certain therapies might require the need for regulatable gene delivery (chapters 5 and 6). This information was used to create suitable vectors expressing Cre recombinase and galanin.
Chapter 7

Applications

7.1. MATERIALS AND METHODS.


7.1.1.1 DRG Tissue Sectioning.

DRG were extracted and fixed as detailed in section 2.2.8.1. The DRG were equilibrated in 30%sucrose/1xPBS at 4°C in order to cryoprotect. The DRG were then mounted in OCT (Histological Equipment Ltd, Nottingham, UK), and sectioned to 15μm on a cryostat at -20°C. The sections were mounted onto slides. The sections were allowed to air-dry for 30mins and then they were analysed for reporter gene expression. For GFP expression the slides were placed directly under a fluorescent microscope (Zeiss axiophot) and observed at a wavelength of 520nm. For β-galactosidase expression slides were put directly into X-Gal solution (5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆.6H₂O, 1mM MgCl₂, 0.02% sodium deoxycholate, 0.02% NP-40, and 40mg/ml X-Gal [dissolved in DMSO] in 1 x PBS) and left overnight at 37°C. Slides were then washed twice in 1x PBS and counterstained with neural red (see section 2.2.8.5.).

7.1.1.2. Genomic DNA Preparation.

Tail snip biopsy was performed on 3 week old ROSA26-R mice (Technical services, UCL, London) in order to obtain genomic DNA. DNA was prepared using a genomic DNA extraction kit (Qiagen, Chatsworth, USA) and was performed following manufacturer's instructions.
7.1.1.3. Genotyping of ROSA26-R mice by PCR Analysis.

PCR analysis was performed on genomic DNA of ROSA26-R mice in order to determine their genotype. In each case two PCR reactions were performed using two different sets of primers.

**Primers**

315 5'- GGG AAG AGT TTG TCG TCA ACC-3'
316 5'- GGA GCG GGA GAA ATG GAT ATG-3'
883 5'- AAA GTC GCT CTG AGT TGT TAT-3'

**Reaction components**

A.  
- dH2O
- 10X Buffer
- Primers 316: 1.2µl
- Primers 883: 1.2µl
- Taq Polymerase: 0.6µl
- 25mM MgCl2: 1.8µl
- 2.5mM dNTPs: 1.2µl
- Tail Snip DNA: 1µl

B.  
- dH2O: 20.6µl
- Primers 315: 1.2µl
- Primers 883: 1.2µl
- Taq Polymerase: 0.6µl
- 25mM MgCl2: 1.8µl
- 2.5mM dNTPs: 1.2µl

**PCR cycling**

- 1x 94°C 1min
- 40x  
  - 94°C 1min
  - 63°C 30sec
  - 72°C 2min
- 1x 72°C 10min

<table>
<thead>
<tr>
<th>Band Profile</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>250bp band only</td>
<td>Homozygous ROSA26-R</td>
</tr>
<tr>
<td>250bp + 500bp</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>500bp band only</td>
<td>Homozygous Wild Type</td>
</tr>
</tbody>
</table>
7.1.2. Galanin Immunohistochemistry.

7.1.2.1. DRG Sections.
2 days following vector inoculation mice were perfused in PBS and then 4% PFA/1xPBS. DRG L4 and L5 were removed and post fixed in 4% PFA/1xPBS for 4hrs. DRG were equilibrated in 20% sucrose/1xPBS at 4°C in order to cryoprotect. DRG were then embedded in OCT (Histological Equipment Ltd, Nottingham, UK) on dry ice and cut on a cryostat to 14μM sections. The sections were mounted onto slides. The sections were blocked with 10% normal serum (goat) (GIBCO, Paisley, Scotland.) in 1xPBS/0.2% Triton-X-100 (TX-100) for 1hr at room temperature. The sections were incubated with primary antibody, rabbit anti-galanin (Affiniti Bioreagents Inc., Colorado, USA) diluted 1 in 1000 in 1xPBS/TX-100 overnight at 4°C. The sections were washed X3 for 10mins in 1xPBS and incubated in secondary antibody, goat anti rabbit FITC (Sigma, Poole, Dorset, UK.) diluted 1 in 200 for 4hrs at room temperature. The sections were washed for X3 for 10mins in 1xPBS and mounted in Vectorshield mounting media (Vector Labs). Sections were then visualised under UV light at a wavelength of 515nm.
7.2. RESULTS.

7.2.1. Vectors Expressing Cre Recombinase.

7.2.1.1. The ROSA26-R Cre Reporter Strain.

The ROSAβgeo26 (ROAS26) mouse strain was produced by random retroviral gene trapping in embryonic stem cells (Friedrich and Soriano 1991). This mouse strain has a β-geo fusion cassette, (a fusion between genes coding for β-galactosidase and Neomycin resistance) inserted at the ROSA26 locus, a locus that is reported to code for three transcripts, only one of which is thought to code for a protein (Zambrowicz et al. 1997). This is a particularly important gene trap strain since expression of the β-geo reporter gene appears to be expressed at all stages of embryonic development and in all tissues (Zambrowicz et al. 1997) (Friedrich and Soriano 1991).

The ROSA26 strain has been used to develop derivatives such that there are now transgenic lines available that can be used for monitoring Cre expression, by targeting of floxed reporter cassettes to the ROSA26 locus (Soriano 1999) (Mao et al. 1999) (Mao et al. 2001). The ROSA26 reporter stain used here, ROSA26-R has been described previously (Soriano 1999). This strain consists of a lacZ excision activation cassette inserted at the ROSA26 locus, expression of lacZ occurs only following Cre mediated excision of an upstream floxed neomycin resistance gene. See figure 7.1.
Figure 7.1. Schematic diagram of the ROSA26 locus and the ROSA26 reporter construct. The ROSA26 reporter construct consists of a splice acceptor sequence, SA, a neomycin expression cassette flanked by loxP sites and a lacZ gene and polyadenylation sequence, bpA. The neomycin resistance cassette consists of a phosphoglycerate kinase-1 promoter, PGK, a neomycin resistance gene, neo, and pA sequence. A triple polyadenylation sequence was added to the 3' end of the neo cassette in order to prevent transcriptional read through. Expression of lacZ only occurs following Cre recombinase mediated excision of the neo expression cassette.
7.2.1.2. Genetic Analysis of ROSA26-R Mice.

A breeding colony of ROSA26-R mice was established. Tail biopsy was performed on 3-week-old offspring and genomic DNA extracted, see protocol 7.1.1.2. PCR analysis on genomic DNA was performed in order to determine the genotype of the animals, see protocol 7.1.1.3. and figure 7.2. Both homozygous and heterozygous ROSA26-R mice were used for further experiments.

7.2.2. A Replication Competent Vector Expressing Cre Recombinase.

7.2.2.1. Cloning Strategy.

It was decided to construct a replication competent vector that expressed Cre recombinase. Previously a vector deleted for ICP34.5/OrfP and inactivated for VP16 and UL43 was shown to give efficient gene delivery to peripheral ganglia, following both footpad and sciatic nerve inoculation, see chapter 3 (Palmer et al. 2000). In particular it was found that a vector containing the expression cassette pR20.5/UL43, gave high levels of transgene expression following sciatic nerve inoculation during the acute stages of viral infection, see section 3.2.3. Since it will be desirable to deliver Cre to large number of cells but only in the short term, it was decided sub-clone Cre recombinase into the pR20.5 cassette in place of lacZ. The advantage of this strategy is that the expression cassette now expresses GFP and Cre recombinase and thus GFP expression can be used to monitor the expected sites of Cre mediated excision events.

pR20.5/UL43 was digested with BamHI/T4 to release a lacZ-pA fragment. pPGK-Cre (John Wood, UCL, London) was digested with XhoI/T4 to release a Cre-pA fragment and this fragment was inserted into pR20.5/UL43 in place of lacZ. Clones were verified by restriction analysis with XbaI. The pR20.5/UL43/Cre expression cassette expresses GFP and Cre recombinase. See figure 7.3.
Chapter 7 Applications

Figure 7.2. PCR genotyping of ROSA26-R mice. Heterozygote ROSA26-R mice were bred and tail biopsy was performed on 3 week old offspring in order to determine their genotype. Two PCR reactions were performed using two primer sets, A and B. See protocol 7.1.1.3. Wild-type mice give a 500bp band with primer set A and nothing with set B. Homozygous ROSA26-R mice give a 250bp band with primer set B and nothing with set A. Heterozygote mice give a 500bp band with primer set A and a 250bp band with primer set B. Results here show the genotype of one litter. Mouse3 is wild-type, mice 2 and 6 are homozygous ROSA26-R, mice 1,4,5,7,8 are heterozygous.
A. 1764

ICP34.5

UL43 Insertion site
NsiI
HSV nt
94911

ICP34.5

UL43

RSV

LATP2

CMV

GFP

pA Cre Recombinase

B. pR20.5/UL43Cre

Figure 7.3. Cre recombinase expression cassette and replication competent vector backbone. A. 1764 vector backbone deficient in ICP34.5/OrfP and VP16. B. pR20.5/UL43Cre expression cassette showing the insertion site of UL43. RSV, Rous sarcoma virus promoter. LATP2 HSV-1 latency associated promoter (HSV-1 nt 118866-120219). CMV cytomegalovirus promoter. GFP Green fluorescent protein. pA, polyadenylation sequence.
7.2.2.2. Vector Preparation and Characterisation.

The Cre vector was constructed by co-transfection of 1764 viral DNA with pR20.5/UL43Cre DNA that had been linearised with *MunI*. Recombinant green plaques were purified away from the white backbone vector. 6 different plaque isolates were purified. One of these isolates, termed 1764pR20.5/UL43Cre, was grown to a high titre stock. BHK cells were infected at an MOI of 0.1 with this vector and cells harvested 24 hours later and 7.4.

Figure 7.4E shows that the replication competent vector 1764 pR20.5/UL43Cre expresses Cre recombinase.
Figure 7.4. Western blot detection of Cre recombinase from HSV-1 vectors. 27/12/M:4 or BHK cells were grown to 90% confluency and were infected with virus at an MOI 1 or 0.1 respectively. 24 hours post infection cells were harvested and samples prepared. Approximately 20μl of each sample were run on a 10% acrylamide/10%SDS gel alongside 5μl of molecular weight markers. The blot was hybridised with a monoclonal anti-mouse IgG Cre antibody (BAbco, Richmond, California, USA.) diluted 1/1000 A. 1764 pR20.5/UL43 B. 1764 27/-4- pR19lacZ. C. Uninfected BHK cells. D. 3 plaque isolates of 1764 27/-4- pR19Cre E. 1764 pR20.5/UL43 Cre. A band at 38kDa can be seen in lanes D1-3. and E. indicating that the vectors express Cre recombinase.
7.2.2.3. A Replication Competent HSV-1 Viral Vector Expressing Cre Recombinase Shows Efficacy In Vivo.

The replication competent vector 1764 pR20.5/UL43Cre was shown to express Cre \textit{in vitro}, see section 7.2.2.3. The vector was now tested \textit{in vivo} to determine if Cre recombinase expression could mediate conditional genomic alterations in ROSA26-Reporter mice. 2-5\mu l of 2\times10^8 pfu/ml of vector was injected unilaterally into the rear sciatic nerve of both ROSA26-R mice and Balb/c mice. A further control was set up by injection of 2-5\mu l of 2\times10^8 pfu/ml of 1764 virus into the rear sciatic nerve of ROAS26-R mice. 1 week post inoculation ipsilateral DRG were removed and observed under UV light at 520nm to detected GFP expression and then X-Gal stained to determine \beta-galactosidase activity (see protocols 2.2.8.3. and 2.2.8.4.). Results can be seen in figure 7.5.

Figure 7.5 shows that following sciatic nerve inoculation of 1764 pR20.5/UL43Cre, GFP expression can be detected in DRG of both ROSA26-R and Balb/c mice. However, \beta-galactosidase activity could only be detected in ROSA26-R mice. Neither GFP or \textit{lacZ} activity could be detected following injection with 1764 vector into ROSA26-R mice. These results indicate that a HSV-1 vector expressing Cre recombinase specifically mediates genomic alterations in ROSA26-R mice. Contralateral DRG were extracted from ROAS26-R mice injected with 1764 pR20.5/UL43Cre and no GFP or \beta-galactosidase activity could be detected, indicating as expected that Cre remains intracellular. Following injection with 1764 vector into ROSA26-R mice no \beta-galactosidase expression could be observed, indicating that genomic alterations are a direct result of Cre activity and are not related to HSV proteins or the presence of HSV-1 virions in the neurons.
Figure 7.5. In vivo efficacy of a replication competent vector expressing Cre recombinase. 2-5μl of 2x10⁸pfu/ml of a replication competent vector expressing Cre recombinase, 1764 pR20.5/UL43Cre, was injected unilaterally into sciatic nerves of A. ROSA26-Reporter mice B. Balb/c mice. 2-5μl of 2x10⁸pfu/ml of 1764 vector was injected unilaterally into the sciatic nerve of C. ROAS26-R mice. 1 week post inoculation DRG were extracted and GFP expression visualised under a fluorescent microscope at 520nm. The DRG were then fixed in 4%PFA on ice. The DRG were washed in 1xPBS and then X-Gal stained to determine β-galactosidase activity. Yellow arrows indicate corresponding cells.
7.2.2.4. In Vivo Efficiency of a Replication Competent Vector Expressing Cre Recombinase is High but Variable.

It was apparent that the Cre recombinase expression vector, 1764 pR20.5/UL43Cre, produced high levels of Cre recombinase as determined by the high levels of lacZ expression and thus genomic alterations observed, see figure 7.5. However, the efficiency of the genomic excision event was unknown. Thus, vector 1764 pR20.5/UL43Cre was injected unilaterally into the rear sciatic nerve of 6 ROSA26-R mice. 3 days post inoculation DRG were extracted and fixed in 4% PFA, cryoprotected in 30% sucrose and sectioned as detailed in section 7.1.1.1. GFP expression was observed under UV light and lacZ expression following X-Gal staining. Results for matching sections from 3 different animals can be seen in figure 7.6. Quantification was not performed since at this stage the experiment was designed as a quick look see.

Results show that in most cases the presence of vector as determined by GFP expression results in a Cre mediated excision event and thus lacZ expression. However it is also apparent that certain cell bodies are positive for lacZ expression but not for GFP expression, indicating that excision has occurred in the absence of vector. It is probable that this phenomenon is just an artefact and is the result of low level expression of GFP in the cell body under question. Furthermore it is also apparent that certain cell bodies are positive for GFP expression and not lacZ, indicating that here, an excision event has not occurred. The number of cells in which this occurred seemed to be variable between animals. In some animals no such cells were apparent and in other animals possibly 10-20% of cells were lacZ negative. Since both heterozygous and homozygous animals were used in the studies described here, this result could be directly related to the genotype of the animals used. Animals in which a high frequency of lacZ expression is observed are likely to be homozygous ROSA26-R animals. Unfortunately it was not possible to match the animal genotypes with the extracted DRGs. It would be an interesting experiment to test this hypothesis by using groups of either homozygous or heterozygous animals. However this was not possible in this study due to a limited supply of animals and the low frequency of homozygous ROSA26-R births.
Figure 7.6. *In vivo* efficiency of a replication competent virus expressing Cre recombinase. A replication competent vector expressing Cre recombinase, 1764 pR20.5/UL43Cre was injected unilaterally into the sciatic nerve of mice. 3 days post inoculation DRG were extracted, fixed in 4%PFA and cryoprotected in 30% sucrose. The DRG were sectioned at 15μm and mounted on slides. GFP expression was detected under UV light at 520nm and on the same section *LacZ* expression was detected after X-Gal staining. Yellow arrows indicate cell bodies positive for both GFP and *lacZ* expression. White arrows indicate cell bodies positive for GFP expression only. Red arrows indicate cell bodies positive for *lacZ* expression only.
7.2.2.5. Kinetics of Cre Recombinase when Delivered by a HSV-1 Vector.

It was decided to investigate the kinetics of Cre recombinase expression and subsequent enzyme activity. 12 ROAS26-R mice were injected unilaterally into their rear sciatic nerve with 2x10^8 pfu/ml of 1764 pR20.5/UL43Cre. DRG were extracted from 3 mice at 1day, 2days, 3days and 30days post inoculation and examined for GFP expression and lacZ activity. Results can be seen in figure 7.7.

Results indicate that as early as 24hous post vector inoculation, Cre recombinase had been expressed and mediated genomic excision at the ROSA26-R locus and subsequent lacZ expression. Results suggest that at 1day post inoculation both GFP expression and lacZ activity was lower than on days 2 and 3. In addition GFP expression and lacZ activity was similar between DRG extracted 2 and 3 days post inoculation. At 1month post inoculation the number of GFP positive cells had dropped significantly, as would be expected from a vector containing the pR20.5/UL43 expression cassette. See section 3.2.3. However, the number of lacZ positive cells remained similar to the number observed at days 2 and 3-post inoculation. Since lacZ expression is produced as the result of a genomic alteration it would be expected to remain unchanged. These experiments should be repeated using a larger number of animals in order to determine the exact kinetics of Cre mediated genomic excision.

Previously a Cre recombinase adenoviral expression vector has been constucted (Stec et al. 1999). This vector was used to mediate a liver specific loss of the angiotensinogen hAGT gene following i.v. inoculation of vector. The group monitored the time course of the Cre-mediated excision event by obtaining plasma samples before and each day after Ad administration. The group found that maximal excision had occurred between 3 and 5 days post vector administration, as determined by the gradual loss of circulating plasma hAGT up to that time and starting at day1. After 3-5 days the loss was shown to be A) 90% and B) undetectable above background, in two of the transgenic lines studied. Like the results presented here, the group did not monitor time point earlier than 24hours. It would be interesting to see here the earliest time
point of Cre mediated excision. *In vitro* it has been shown that a HSV-1 vector expressing Cre recombinase is capable of mediating an excision event from a transiently transfected plasmid as early as 6 hours post infection. (Rinaldi *et al.* 1999).
Figure 7.7. Kinetics of Cre recombinase activity when delivered by a HSV-1 vector. 2.5 μl of 2x10^5 pfu/ml of vector 1764 pR20.5/UL43Cre was injected unilaterally into the rear sciatic nerve of ROAS26-R mice. A. 1 day, B. 2 days, C. 3 days, and D. 30 days post inoculation DRG were extracted and observed for GFP expression under UV light at 520nm and for lacZ activity following X-Gal staining.
7.2.3. A Replication Incompetent Vector Expressing Cre Recombinase.

It was found previously that a replication incompetent vector deficient in ICP34.5/OrfP, ICP27, ICP4 and VP16 was capable of high-level gene delivery to peripheral ganglia, see section 4.1.2. (Palmer et al. 2000). Thus, it was decided to construct a replication incompetent vector containing Cre recombinase. It was hypothesised that such a vector would be less toxic to cells because it is deficient in IE gene expression (Lilley et al. 2001). On non-complementing cells the only HSV-1 protein this vector expresses to significant levels is ICP6 (Lilley et al. 2001), which has been shown to be non-toxic to cultured cells (Johnson et al. 1994).

7.2.3.1. Cloning Strategy and Vector Preparation.

pR19CMVlacZ (laboratory plasmid, UCL, London) was digested with HindIII/T4/Xbal to release a lacZ-pA fragment. pGK-Cre (John Wood, UCL, London) was digested with SalI/T4/Xbal to release a Cre recombinase fragment. The Cre fragment was cloned into pR19CMVlacZ in place of lacZ. The resulting plasmid was called pR19Cre. The vector was constructed by co-transfection of 1764 27-/4- pR19CMVlacZ viral DNA with pR19Cre linearised with Scal. Recombinant white plaques were purified away from blue vector backbone. The resulting vector was called 1764 27-/4-pR19Cre. See figure 7.8. Three different plaque isolates were purified and grown to a MS following protocol 2.2.6.2.

All 3 plaque isolates were tested for Cre recombinase expression by western blot analysis. 27/12/M:4 cells were infected at an MOI of 1 with each stock of vector. In addition 27/12/M:4 cells were infected with 1764 27-/4-pR19CMVlacZ at an MOI of 1 as a negative control. Samples were harvested and processed following protocol 2.2.7.1. A monoclonal anti-mouse Cre recombinase antibody was used and diluted 1 in 500 (BAbco, Richmond, California, USA). Results can be seen in figure 7.4D and E. Western blot analysis shows that all three isolates, 1-3, express Cre recombinase. Isolate number 1 was chosen for further experiments.
Figure 7.8. Schematic representation of Cre recombinase and galanin expression cassettes and replication incompetent vector backbone. A. 1764 27-14- vector backbone deficient in ICP27, ICP4, ICP34.5/OrfP and VP16 and showing insertion sites in the LAT regions. See section 7.2.3. for details. B. pR19Cre expression cassette. C. pR19Gal expression cassette. The cassettes were cloned into the HSV LAT region in a 3.5kb NotI fragment cloned into pGem5 (HSV-1 nt 118441 to 12202). LAP1 and LATP2, latency associated promoters. CMV, cytomegalovirus promoter. pA, polyadenylation sequence.
7.2.3.2. A Replication Incompetent HSV-1 Vector Expressing Cre Recombinase Shows Efficacy In Vivo.

The replication incompetent vector 1764 ICP27-/ICP4- pR19Cre was shown to express Cre recombinase \textit{in vitro}, see section 7.2.3.2. The Vector was then tested for efficacy \textit{in vivo}. 2-5\mu l of $3 \times 10^5$ pfu/ml of vector was injected unilaterally into the rear sciatic nerve of 3x ROSA26-R and 3x Balb/c mice. In addition 2-5\mu l of $3 \times 10^5$ pfu/ml of vector 1764 27-/4- pR19GFP was injected unilaterally into the rear sciatic nerve of 3x ROSA26-R mice. 3 days post inoculation DRG were extracted and examined for GFP expression and/or fixed in 4%PFA on ice and X-Gal stained to determine \textit{lacZ} activity. Results can be seen in figure 7.9.

Results show that as expected \textit{lacZ} activity was only detected in DRG from ROSA26-R mice that had been injected with 1764 27-/4- pR19Cre. ROSA26-R mice injected with 1764 27-/4- pR19GFP showed no \textit{lacZ} activity. This indicates that like the results observed with the replication competent vector, conditional genome alteration is mediated by Cre recombinase and is not a function of an HSV-1 protein or infection.
Figure 7.9. *In vivo* efficacy of a replication incompetent vector expressing Cre recombinase. 2-5µl of 3x10^6pfu/ml of a replication incompetent vector expressing Cre recombinase, 1764 27-14-pR19Cre, was injected unilaterally into the rear sciatic nerve of mice. A. ROAS26-Reporter mice and B. Balb/c mice. 2-5µl of 3x10^6 pfu/ml of 1764 27-14-pR19GFP vector was injected unilaterally into the rear sciatic nerve of C. ROAS26-R mice. 1 week post inoculation DRG were extracted and GFP expression visualised under a fluorescent microscope at 520nm and then X-Gal stained to determine β-galactosidase activity.
7.2.4. A Viral Vector Expressing the Neuropeptide Galanin.

It was decided to construct a disabled HSV-1 vector expressing galanin. A non-cytotoxic HSV-1 vector will be required for rescuing the phenotype of knockout animals in vitro, since the replication competent vectors described in chapter 3 have been found to be considerably toxic to primary DRG cultures, whereas vectors deficient in IE gene expression are not (Caroline Lilley, personal communication (Lilley et al. 2001)). Thus a vector deleted for ICP34.5/OrfP ICP27, ICP4 and VP16 was chosen as a vector backbone. See section 4.2.1. In vivo galanin expression will be required over a long period in order to aid regeneration in the knockout animals, thus again the replication incompetent backbone will be suitable.

7.2.4.1. Cloning Strategy and Vector Production.

Plasmid pKS/mGalanin-Bam (Niall Kerr, University of Bristol, Bristol) was digested with NotI/T4/EcoRI to release a 4731bp galanin fragment. This was a mouse galanin genomic locus EcoRI fragment minus a 5'BamHI fragment containing the promoter. pR19CMV/lacZ (laboratory plasmid, UCL, London) was digested with HindIII/T4/EcoRI to release a lacZ fragment. The genomic galanin fragment was sub-cloned into pR19CMV/lacZ in place of the lacZ fragment to produce pR19GalGen. See figure 7.8.

The vector was constructed by co-transfection of 1764 27-/4- pR19CMVGFP viral DNA with green vector backbone. The resulting vector was called 1764 27-/4-pR19GalGen. See figure 7.8. Six different plaque isolates were purified and grown to a MS following protocol 2.2.6.2.

All 6 plaques isolates were tested for the presence of galanin genomic DNA by southern blot analysis. 27/12/M:4 cells were infected at an MOI of 1 with each stock of vector. In addition 27/12/M:4 cells were infected with 1764 27-/4-pR19CMV/lacZ, a vector not expressing galanin, at an MOI of 1 as a negative control. 24 hours post infection samples were harvested and viral DNA prepared for southern blot analysis following protocol 2.1.3.3. DNA was digested with XhoI. Southern blot analysis was performed following protocol 2.1.7. Plasmid pKS/mGalanin-Bam was digested with EcoRI/XhoI to release
an ~1kb 3’ galanin genomic fragment and this fragment was used as the probe. pR19GalGen was digested with Xhol, to release an ~1.6kb galanin fragment and this was used as the positive control on the gel. Results can be seen in figure 7.10. Southern blot analysis showed that 5 out of 6 purified plaques contained galanin genomic DNA. Isolate number 4 was chosen for further experiments.
Figure 7.10. Southern blot detection of galanin genomic DNA in recombinant viral vector genomes. Recombinant viral vectors were constructed containing galanin genomic DNA. Viral DNA was checked for the presence of Genomic DNA by southern blot analysis. A. plasmid pR19GalGen, positive control B. Vector 1764 27-4- pR19CMV GFP, negative control C. 6 plaque isolates thought to contain galanin genomic DNA. All DNA was digested with XhoI. Galanin DNA should release a 1.6kb fragment upon XhoI digestion. The blot was probed with a 1kb fragment from pKS/mGalanin-Bam digested with EcoRI/XhoI. Plaques isolates 2-6 were positive for Galanin genomic DNA.
7.2.4.2. Ex Vivo Detection of Galanin Expression from a HSV-1 Vector.

Following Southern blot analysis of vectors containing galanin genomic DNA, it was necessary to check whether vectors expressed a functional protein. Since Galanin is a neuropeptide of 29 amino acids western blot analysis was not possible. Thus, the vector was verified by immunohistochemical detection of galanin on DRG sections from animals that had been previously injected with vector 1764 27/-4-pR19GalGen. Vector 1764 27/-4- pR19CMVlacZ was used as a negative control.

10μl of 2x10^8 pfu/ml of each vector was injected unilaterally into the rear sciatic nerve of galanin knockout animals. 3 days post inoculations DRG were extracted, sectioned and processed for galanin expression following protocol 7.1.2.1. Results can be seen in figure 7.11. Immunohistochemistry was also performed on DRG sections in the absence of primary antibody (galanin). These sections were used as a further negative control. The positive controls used were DRG sections from a wild-type animal that had undergone axotomy 1 week prior to DRG extraction. Following nerve injury endogenous galanin levels increase by 120-fold in the adult (Hokfelt et al. 1987). Thus, in these animals galanin levels would be the normal amount following nerve injury. Results can be seen in figure 7.11.

It is known that in adult rat DRG, galanin is normally expressed in only 2-3% of DRG cells which are predominantly small fibre neurons (Hokfelt et al. 1987). Following axotomy mRNA and peptide are abundantly expressed in 40-50% of all DRG neurons (Hokfelt et al. 1994). Results in figure 7.11A show endogenous galanin levels following axotomy in a wild-type mouse and thus shows the expected galanin levels following nerve injury. Figure 7.11B shows a DRG section from a galanin knockout mouse that was injected with 10μl of 2x10^8 pfu/ml of vector 1764 27/-4- pR19GalGen. A number of immuno-positive cells can be detected although it became apparent that the numbers of positive cells were not as high as seen in the axotomised animal (Fiona Holmes, personal communication). Figure 7.11C shows a negative control and is the same as 7.11B but the sections were incubated without primary antibody. Here no positive cells can be seen. Figure 7.11D shows DRG sections from an
animal that had been injected with the control virus 1764 27/-4- pR19CMVlacZ. Here no immuno-positive cells can be seen indicating that virus infection is not responsible for the galanin immunoreactivity.
Figure 7.11. *Ex Vivo* Immunohistochemistry to detect galanin expression from a recombinant HSV-1 vector in DRG cells.

DRG from animals were extracted, sectioned and processed by immunohistochemistry for detection of galanin expression. An FITC conjugated secondary antibody was used and positive cells were visualised at 515nm.

A. DRG section from a wild-type mouse 7 days post-axotomy.

B. DRG section from a galanin knockout mouse, 3 days post inoculation with 10 μl of 2 x 10⁵ pfu/ml of 1764 27-4- pR19GalGen.

C. As for B. but sections incubated without primary antibody.

D. DRG section from a galanin knockout mouse, 3 days post inoculation with 10 μl of 2 x 10⁵ pfu/ml of 1764 27-4- pR19CMVlacZ.
7.3. DISCUSSION.

This section of work reports the successful construction of both replication competent and replication incompetent HSV-1 vectors expressing Cre recombinase. Cre recombinase is a 38kDa protein from P1 bacteriophage that mediates recombination events in DNA sequences that are located between loxP sites (Sternberg and Hamilton 1981). A number of viral vectors have been previously reported that express Cre recombinase and have been used successfully to direct conditional transgene expression in isolated pancreatic islets (Piston et al. 1999), in the hippocampus (Brooks et al. 1997), and liver, (Stec et al. 1999) of transgenic mice.

Work here describes the construction of a replication competent HSV-1 vector deficient in ICP34.5/OrfP and VP16, which expresses Cre recombinase. Following sciatic nerve inoculation of vector, Cre was shown to be functional as early as 24hours-post inoculation. Earlier time points were not studied but should be considered for future work. The efficiency of vector appears to be extremely high as judged by the number of cells concomitantly expressing GFP and lacZ. It is thought that the efficiency of excision is directly related to the genotype of the animal. Homozygous ROSA26-R animals will have more chance of Cre mediated gene excision than will heterozygous animals. This hypothesis should be investigated and if proved correct then experiments relating to voltage gated sodium channels should be performed in homozygous ROSA26-R animals only. Experiments of this nature will require a large breeding colony and was thus beyond the scope of the work presented here.

The efficiency of vectors expressing Cre recombinase has been monitored previously. The Ad/Cre expression vector described earlier in section 7.0. (Stec et al. 1999) was found to be 90-100% efficient in reducing circulating levels of hAGT, but authors found this discovery hard to reconcile with the fact that only 50-75% of DNA was converted to the defective form by Cre recombinase (as estimated by southern blot analysis). The authors suggested that the reason for this was that each cell contained multiple copies of the integrated transgene and thus incomplete copies or re-arrangements of the transgene were produced. Thus making the observed efficiency by southern blot lower than
expected by expression data. This however highlights that in transgenic animals 100% efficient excision is not necessary to bring about a significant pharmacological change.

Work here also describes the generation of a replication incompetent vector deficient in ICP34.5/OrfP, VP16, ICP27 and ICP4, which expresses Cre recombinase 1764 27-14- pR19Cre. This vector was shown to be effective in vivo. It will be difficult to easily determine the efficiency of this vector since the virus does not express a marker gene, as seen in the replication competent vector containing the pR20.5/UL43 expression cassette. However this vector has the advantage of a reduced cytoxicity since it expresses minimal levels of IE proteins (Lilley et al. 2001).

Since it is hoped that Cre expression vectors will be used for the conditional gene knockout of SNS or NaN sodium channels, it will be imperative that the vectors efficiently reach small diameter sensory neurons. At this point it unclear whether the vectors described here infect all diameter neurons with equal efficiency, or show a size preference. The next step will be to determine which diameter neurons the vector infects and if small diameter neurons are targeted, what percentages are targeted. This work is currently being undertaken.

Two previous reports have detailed the construction of HSV-1 vectors expressing Cre recombinase. One of these was a HSV-1 amplicon that expresses Cre from the IE ICP22 promoter and uses an ICP4-deleted HSV-1 mutant as helper (Brooks et al. 1997). The second report was of a disabled HSV-1 vector expressing Cre recombinase that was deficient in IE gene expression (Rinaldi et al. 1999). The vector was impaired for VP16, ICP0 and ICP4 gene expression (Preston et al. 1998). Rinaldi et al adopted a similar strategy to the one described here, by the construction of HSV-1 vectors deficient in IE gene expression. They co-injected two vectors into the mouse footpad, one expressing Cre and the other containing a floxed reporter construct. They reported that per mouse the average number of lacZ positive neurons in 7 animals was 33. This relied on the co-infection of the same cells with both vectors. These results showed that a Cre expressing HSV-1 vector
deficient in IE gene expression was functional in vivo. However, their mutant vector in1312, which was used to create the vectors described, contained a temperature sensitive ICP4 mutation, the non-permissive temperature being 38°C. As discussed previously (chapter 4) it is likely that the mouse footpad is somewhat below the non-permissive temperature and thus it is possible that replication and IE gene expression would occur in this model, rendering the vector more cytotoxic than expected. In contrast to this a vector similar to the replication incompetent vector described here, but expressing lacZ and not Cre has been shown to be non-toxic in the CNS and direct lacZ expression up to 28 days, the longest time point tested (Lilley et al. 2001). This would suggest that the replication incompetent vector 1764 27-l4-pR19Cre would be suitable for use in the CNS. This could be tested in the future in ROSA26-R mice.

Work in this section also describes the generation of a replication incompetent vector deficient in ICP34.5/OrfP, VP16, ICP27 and ICP4, 1764 27-l4-pR19GalGen that expresses the neuropeptide galanin (Tatemoto et al. 1983). Immunohistochemistry suggests that the vector efficiently expresses galanin in vivo. It is hoped that the vector will be used to rescue the phenotype of a galanin knockout animal. Thus, in vivo the vector will cause an increase in nerve regeneration following nerve injury and in vitro increase neurite outgrowth in primary DRG cultures from knockout mice. In vitro studies will require the infection of DRG cultures 1 day post plating. Subsequent to the generation of this vector it has been found that DRG cultures are transduced more efficiently when left for a week before infection (Caroline Lilley, personal communication). However in the model described here, it is essential to infect DRG cultures 1 day post plating, otherwise it is impossible to measure neurite outgrowth. Work is currently being undertaken to see if infection efficiencies 1 day post plating are sufficient to see a phenotype rescue.

It has previously been reported that HSV infection of peripheral ganglia leads to an up-regulation in galanin positive neurons (Henken and Martin 1992b; Henken and Martin 1992a). In these reports galanin levels were examined both in the DRG following footpad inoculation with HSV-2 (Henken and Martin 1992a) and in trigeminal ganglia following corneal sacrifice with HSV-1 (Henken and
Martin 1992b). Both reports concluded that HSV infection lead to a transient and reversible increase in neuronal galanin content in ganglia of BALB/c mice. The group suggested that this phenomenon was possibly a result of damage to or death of neuronal and/or nonneuronal cells in sensory ganglia. Galanin up-regulation may then be a response to this trauma and be involved in nerve regeneration. Whilst this is an interesting phenomenon, these finding will not effect the experiments here since the mouse model used is a galanin knockout, thus galanin expression will only arise from that encoded in the recombinant vector genome. In addition the HSV strains used by Henken et al (Henken and Martin 1992a; Henken and Martin 1992b) were both wild-type and thus likely to cause nerve damage and/or death. Vectors described here are replication incompetent and non-toxic (Lilley et al. 2001) and thus are highly unlikely to cause cell damage or death. However, it would be interesting to see if infection with such vectors would cause an increase in galanin immunoreactivity in mice.

In conclusion, this chapter describes the construction and verification of vectors expressing functional Cre recombinase and galanin. Clarification of which population of sensory neurons the Cre recombinase vector infects will be required before further use of the virus. The next step is to use these vectors in the studies of voltage gated sodium channels and nerve regeneration, in the two model systems described.
CHAPTER 8:

DISCUSSION
CHAPTER 8:
DISCUSSION

Herpes simplex virus is potentially an excellent tool for gene therapy in the nervous system for a number of reasons. HSV-1 is neurotrophic and since the virus life cycle involves infection of peripheral nerves, the virus seems an ideal choice for developing as a vector for gene therapy in the PNS. The virus also has the ability to enter a long, often life-long, period of latency in the nuclei of peripheral ganglia. This provides a platform from which long-term transgene expression might be achieved. Most disease pathologies, for example Parkinson's disease and Alzheimer's disease, mean that treatment will require long-term therapy. Therefore the successful achievement of long term gene expression is central to the development of most gene therapy strategies. Furthermore, HSV has evolved the ability for retrograde transport along axons such that gene delivery to ganglia can be achieved following non-invasive peripheral administration, a feature not efficiently possible with other viral vectors. In addition the HSV -1 genome is large and hence provides a greater insertional capacity for foreign genetic material compared to other smaller viruses. This is an important feature since many disease treatments are likely to require the delivery of multiple genes for maximum efficacy. For these reasons, we and others have chosen to develop HSV-1 as a vector for gene delivery in the nervous system.

At present there are some unanswered questions regarding the necessary properties that HSV must retain in order to act as an efficient gene delivery vector. The work presented in this thesis aimed to answer some of these questions and describes the development of HSV-1 vectors for gene delivery to the PNS.

Work in chapters 3 and 4 was aimed at identifying parameters and gene deletions from the HSV genome that allow gene delivery to peripheral ganglia. This resulted in the identification of optimal replication competent and incompetent gene delivery vectors. Chapters 5 and 6 were concerned with the development of gene delivery vectors from which gene expression could be regulated. An effective regulatable system would ideally have low basal
expression in the uninduced state, have rapid induction kinetics that can be quickly and efficiently reversed, should not interfere with endogenous factors and the ligand (through which gene expression is regulated) should be safe and easy to administer. Systems such as this would not only greatly enhance the safety profile of gene therapy protocols, but would provide a means whereby gene function in the nervous system could be more easily studied. Chapter 7 was concerned with the use of the developed vectors in two model systems, to deliver Cre recombinase for Cre/loxP mediated regulation of gene expression and to study nerve regeneration mediated by the neuropeptide galanin.

Results in chapter 3 identified that a vector deficient for ICP34.5/OrfP and VP16 (1764) gave efficient gene delivery to peripheral ganglia during lytic and latent infection, following both footpad and sciatic nerve inoculation. A vector that was further deleted for vhs was shown to give less efficient gene delivery. The level of transgene expression from various 1764-based vectors was dependent upon the expression cassettes that were inserted. A number of expression cassettes were studied that were either located in the endogenous LAT region, (pR19CMVlacZ or pR19MMLVlacZ) or in the nonessential genes US5 and UL43, (pR20.9 and pR20.5).

Cassettes pR19CMVlacZ and pR19MMLVlacZ were inserted in the LAT region, 3' to LATP2, in order to link the CMV or MMLV promoters to the endogenous LAT promoter elements. LATP2 was identified in our laboratory (Palmer et al. 2000) and includes the elements of LAP2 promoter that have previously been described (Goins et al. 1994). LAP1 is the only promoter known to be active during latency, giving rise to a set of latency associated RNA transcripts. To a greater or lesser extent, promoter systems including elements from LAP1 and/or LAP2 have been shown to be capable of supporting transgene expression during latency, either alone or in conjunction with other promoters (Goins et al. 1994; Lokensgard et al. 1994; Lokensgard et al. 1997; Berthomme et al. 2000). The vectors developed in chapter 3 of this thesis (1764 pR19CMVlacZ and 1764 pR19MMLVlacZ), were shown to drive latent transgene expression at least 6 months p.i., but which declined over time. The vector containing pR19CMVlacZ proved to be efficient following both footpad
and sciatic nerve inoculation, whereas the vector containing pR19MMLVLTR/lacZ was only efficient following footpad inoculation. These results suggested that the LAP1 and/or LATP2 promoters were able to aid latent transgene expression from these cassettes inserted directly in the endogenous LAT regions. The levels of transgene expression were directly related to the promoter choice and route of inoculation.

The pR20.9 and pR20.5 expression cassettes both contained a central LATP2 region and a pair of promoters located in a back-to-back orientation driving the expression of two reporter genes, lacZ and GFP (see figure 3.3). Vectors 1764 pR20.9/UL43, 1764 pR20.9/US5 and 1764 pR20.5/UL43 were constructed and the gene expression profile from the two cassettes was unexpectedly different, especially following sciatic nerve inoculation. The position of the pR20.9 cassette, either in UL43 or US5 loci, appeared to make no difference to the levels of transgene expression observed. In conclusion following footpad inoculation, the pR20.9/UL43 cassette was found to give reasonable levels of transgene expression that persisted up to 6 months, although at later time points levels were lower than during lytic infection. The pR20.5 cassette was found to give reasonable levels of transgene expression during lytic infection that declined to low levels at later time points. Conversely, and following sciatic nerve inoculation, the pR20.5 cassette produced high levels of both GFP and lacZ expression during acute infection but which again declined during latent time points. The pR20.9 cassette gave both lacZ and GFP expression that increased during latent infection compared to lytic infection, as would be expected from latency associated promoters LAP1 and LATP2. The difference in gene expression patterns produced by the two cassettes, pR20.9 and pR20.5 makes the vectors suitable for different applications and will be discussed later.

Since transgene expression from most of the vectors studied in this thesis declined during latent infection, the extent to which this was related to any immune response following vector administration and/or to any shut down of promoters at later time points was investigated. Experiments using the immunomodulatory drug cyclosporin did not increase transgene expression compared to that observed in immune competent mice. However these results
do not preclude the possibility that increasing either the dose of cyclosporin, or the frequency of administration might improve transgene expression, as has now been seen in a hamster model with a HSV vector (Mabon et al. 1999).

Experiments performed recently in our laboratory suggest that by using SCID mice, which lack B and T lymphocytes, transgene expression from the pR20.9 cassette can be improved during latent infection, but not to the same levels seen in lytic infection (unpublished data). This indicates that an immune response is at least partly responsible for the decline in transgene expression that is observed. Further experiments using different immune-suppressive drugs at various dosages may be beneficial in studying this observation. However in a clinical situation immunosuppression of patients would not be preferable.

*In situ* hybridisation (ISH) for LAT RNAs was performed to investigate to what extent promoter shut-off was responsible for the drop in transgene expression during latency. Results showed that vectors that did not express any transgene, (1764 and 1764 P2NSELacZ/UL43) showed an increase in the amount of LAT production during latent infection compared to lytic infection, and the number of LAT positive cells remained consistent over time (see figure 3.14). During latent infection vectors containing either the pR20.9 and pR20.5 cassettes produced similar numbers of LAT positive cells and LacZ-positive cells. In these vectors the number of LAT positive cells after 1 month was reduced compared to the non-marker gene containing viruses, indicating that possibly immune responses to the transgene, rather than promoter shutdown, were responsible for the drop in transgene expression observed.

Collectively these results suggest that elements within the ~1.4kb LATP2 region are capable of conferring latent transgene expression on the MMLVLTR and CMV promoter and the LAP1 promoter itself. This was evident in the endogenous LAT region and at ectopic sites within the genome. However it is possible that elements other than those located in the LATP2 region, perhaps elements in LAP1, are involved in latent transgene expression since the pR20.5 cassette, containing LATP2, was less effective than pR20.9 at giving latent gene expression.
The vectors described in chapter 3 report for the first time vectors that express two heterologous genes during HSV-1 latency. Since many disease pathologies will require treatment with multiple factors, then gene therapy vectors capable of this will be necessary. Moreover vectors that express multiple genes from the same locus, again like those described here, will be simple to construct and will have advantages over vectors requiring more complex manipulation.

Work in chapter 4 described the development of replication incompetent vectors for gene delivery to peripheral ganglia. Results identified that a vector deficient for ICP34.5/OrfP, VP16, ICP27 and vhs gave inefficient gene delivery to peripheral ganglia. This was following both sciatic nerve or footpad inoculation when used at the titres that gave high-level gene expression from replication competent vectors (1x10^7 pfu/ml and 1x10^8 pfu/ml respectively). However, it was hypothesised, following subsequent results with vectors additionally deleted for ICP4, that if used at higher titres, an improved gene delivery profile would be observed. This is something that could be investigated in the future. However in light of the successful results presented in chapter 4, using further disabled vectors, it is unlikely that this will be necessary.

A fully replication incompetent vector deficient in ICP34.5/OrfP, VP16, ICP27 and ICP4 and containing a pR19CMV/ lacZ expression cassette in the endogenous LAT region was created. It was found that this vector gave low efficiency gene delivery when used at low titre, but when used at a high titre (5x10^8 pfu/ml) and following sciatic nerve inoculation it was capable of gene delivery to peripheral ganglia during both lytic and latent infection. Transgene expression during latent infection appeared to be consistent with that observed during lytic infection, with no apparent drop-off. There has been only one previous report of a replication incompetent vector allowing gene delivery to peripheral ganglia (Dobson et al. 1990). However in this report, gene delivery efficiency was extremely low. The advantage of the vector described in chapter 4 is that it is deficient in IE gene expression and the only gene expressed to significant levels on non-complementing cells is the IE/E gene ICP6 (Lilley et al. 2001). This gene is not thought to be toxic (Johnson et al. 1994). This thesis therefore, reports for the first time a vector (HSV or otherwise) capable of gene
delivery to peripheral ganglia that is maintained in the long term at high and consistent levels.

Following the development of both replication competent and replication incompetent vectors in chapters 3 and 4, the information gained regarding the gene delivery capabilities of each vector was used in the construction of suitable vectors for the expression of Cre recombinase and the neuropeptide galanin. The required expression profile of the two proteins was completely different and thus different vectors would be optimal. Whilst it was necessary for the vector containing galanin to express the neuropeptide at high levels in the long term in order to mediate nerve regeneration, expression of Cre recombinase would only be required in the short term. Once loxP mediated DNA excision had occurred, Cre activity would no longer be required or useful.

Both replication competent and incompetent vectors expressing Cre were constructed. In a 1764 backbone, Cre was inserted into the pR20.5 cassette in place of lacZ. In a replication incompetent vector deficient for ICP34.5/OrfP, VP16, ICP27 and ICP4, Cre was inserted in the pR19 cassette in place of lacZ. Both vectors were found to be efficient in vivo and mediated the conditional expression of lacZ in ROSA26-R mice. The replication competent vector was particularly advantageous since it co-expressed GFP and Cre, the GFP provided a marker for expected excision events and thus provided a simple means by which to determine the efficiency of the vector at causing excision at loxP sites. The efficiency of the vector was shown to be possibly 80% as judged by approximating the number of cells that were positive for both lacZ and GFP in the same section. However, if this was to be quantified further in the future, immunohistochemistry could be performed. The replication incompetent vector may be particularly useful since it is known that vectors with these gene deletions are non-toxic to cultured cells, and thus presumably in vivo, as they do not express IE genes to significant levels.

For the Cre expressing vectors, it will be important to determine which diameter sensory neurons the virus infects. Since it is hoped that these vectors will be used for the conditional knockout of SNS Na+ channels, which are located
preferentially in small diameter sensory neurons, it is essential that this subset of neurons are targeted. Preliminary immunohistochemistry suggests that the HSV/Cre vectors described in this thesis infect all diameter neurons, although the frequency of infection of each is not known. Since in vivo experiments are likely to involve sciatic nerve inoculation, it might be expected that small diameter neurons would be targeted with less efficiency since they are by nature smaller. This remains to be seen.

A replication incompetent vector was constructed deficient for ICP34.5/OrfP, VP16, ICP27 and ICP4, which contained the neuropeptide galanin in the pR19 cassette in place of lacZ. This vector was shown to express galanin both in vitro and in vivo. As mentioned previously, this type of vector is deficient in IE gene expression and the only HSV gene expressed to significant levels on non-complementing cells is the IE/E gene ICP6. The efficacy of this virus in aiding nerve regeneration is to be studied in collaboration with a group at the Bristol University.

Chapters 5 and 6 describe the development of regulatable viral vectors for gene delivery to the PNS. Three systems were studied, the 'Tet-On' system (Gossen et al. 1995), the ecdysone system (No et al. 1996) and the mifepristone system (Wang et al. 1994). Single expression cassettes were created that contained all the elements of each regulatory system, (pR20.4 [Tet-On], pR20.8 [ecdysone], and pR20.11 [mifepristone]), (see figure 5.1). These cassettes, like pR20.9 and pR20.5, contain a central LATP2 element and then two promoters located in a back to back arrangement. Transient transfection assays showed that only the pR20.8 expression cassette gave reasonable levels of ligand inducible β-galactosidase activity, whereas the other cassettes gave less than 5-fold gene induction. It has been speculated that these disappointing results could be a direct consequence of the cell lines chosen for in vitro studies BHK and ND7 cells. This possibility should be investigated further by using other cells e.g. HeLa cells, which was the cell line chosen for development of the original 'Tet-On' and 'Tet-Off' systems.
Replication competent vectors were subsequently created that contained the ‘Tet-On’ (pR20.4) and ecdysone (pR20.8), expression cassettes. These vectors were tested for efficacy both in vitro and in vivo. Results showed that neither vector was regulatable in vitro. Even the ecdysone inducible virus gave poor levels of induction, which was not expected from the results of the transient transfection studies. Subsequent to these findings it has been reported that HSV-1 infected cell proteins ICP4, ICP0 ICP27 and VP16 influence tetracycline regulated transgene expression (Herrlinger et al. 2000). Since a replication competent vector will still express all of these proteins, it is possible that the efficacy of the ‘Tet-On’ and ecdysone vectors was affected by the presence of HSV-1 proteins. This possibility was investigated by the transient transfection of the ecdysone response element, E/GRE\DeltaHSPlacZ followed by superinfection with various HSV-1 vectors with serial gene deletions. The ecdysone response element should not express lacZ unless it is accompanied by the transactivator, VgRXR, and effector i.e. ponasteroneA. Results showed that only a vector that was deficient for ICP34.5/OrfP, ICP27, ICP4 and VP16 did not interact with the ecdysone response element to cause transactivation of lacZ. To find exactly which HSV-1 proteins are involved in interference, double transient transfections could be performed using the ecdysone response element plasmid together with plasmids expressing individual HSV-1 proteins.

The ecdysone inducible vector 1764 pR20.8lacZ/UL43 was tested for efficacy ex vivo. DRG extracted from mice that had been injected with the vector were incubated overnight in FGM either with or without the effector ponasteroneA. Results showed that DRG incubated in the presence of ponA showed much higher levels of lacZ expression than those incubated without ponA, thus indicating that the vector appeared to be functional. It is expected that the replication competent vector described here will produce significantly lower levels of viral proteins in vivo in neurons, than would be expected in vitro. The 1764 vector is deleted for ICP34.5 the neurovirulence factor and thus cannot replicate effectively in neurons. Thus in the in vivo situation, interference of the ecdysone inducible system by HSV-1 proteins is minimised, explaining the improved basal activity and efficacy observed.
Considering the discovery that HSV-1 proteins constitutively transactivate the regulatable systems tested, a replication incompetent regulatable vector in which all HSV transactivators are deleted, or expressed at only very low levels, was constructed. Here, vectors deficient in ICP34.5/OrfP, VP16, ICP27 and ICP4 were constructed that expressed the ecdysone response element and transactivator in the UL43 and US5 loci respectively. Two vectors were created that differed with respect to the response element. One vector contained LATP2 5' to the response element, the other did not. This construction strategy was designed to assess the effects of LATP2 on regulated gene expression. The two vectors were created and tested in vitro and unlike previously these vectors proved to be clearly inducible in vitro, giving up to a 58.4fold induction of β-galactosidase activity. However, in both cases basal transgene expression was observed and it is hypothesised that a residual amount of ICP0 and other HSV-1 proteins were carried over from the complementing cell line and might be responsible for this activity. Interestingly and as expected, the vector that contained LATP2 5' to the response element appeared to give a higher fold induction but higher basal activity than that without the LAT promoter. The reason for this observation is unclear at present, however it is possible that LATP2 could contain binding motifs for IPC0 and/or ICP4 which act to up-regulate its activity.

In vivo and ex vivo analysis of the vectors is inconclusive at this time since it is unclear whether the vectors can successfully reach the ganglia following sciatic nerve inoculation. It has not been previously reported that a vector deficient in ICP34.5/OrfP, VP16, ICP27, ICP4, UL43 and US5 can efficiently reach spinal ganglia following peripheral administration. However, since US5 and UL43 are both non-essential genes then it would seem reasonable to hypothesise, based on previous work (see chapter 4), that such a vector will be capable of such retrograde transport, if inoculated at sufficient titre. This question might be addressed by performing ISH to detect LAT RNA or more simply by dual inoculation studies. The fully disabled ecdysone vectors could be inoculated into animals and left for a week to begin the establishment of latency. Following this a replication competent vector which expresses GFP but no lacZ would be injected into the same nerve, alongside ponA administration. The idea here
being that if the initial vector has efficiently reached the ganglia then the replication competent vector will transactivate otherwise silent promoters in the replication incompetent vector and cause expression of lacZ. However, it has recently been found in the laboratory that for a vector of this level of disablement to give any gene expression following retrograde transport, the promoter used must be linked to LATP2. In the vectors described in chapter 6 the transactivator protein encoding gene is not linked to LATP2, this with hindsight probably explains the lack of regulated gene expression when the vectors were tested in vivo and ex vivo (see section 6.2.1.6. and 6.2.1.7.).

In addition it will be important to determine the bioavailability of ponA, and other ecdysteroids, in the DRG following IP and/or other injection routes. Finally it would be sensible to construct a fully replication incompetent vector containing the pR20.8 cassette in the UL43 locus, as the pR20.8 cassette was the most promising in the context of a replication competent vector. This was not carried out previously because work using replication incompetent vectors began before it was possible to fully characterise the replication competent vector.

Construction of tetracycline repressor gene regulated plasmids has been described in chapter 6. The tet repressor system has the advantage over other systems in that in the uninduced state binding of the tet repressor downstream to the tet operator sites provides a physical block to transgene expression. In the ‘Tet-On’ and ‘Tet-Off’ systems, the response element is located upstream to the tet operator sites and thus no physical block to transcription is achieved. The tet repressor plasmids described here, pR20.1/27/TO/TR and pR19TO/TR/IRESGFP, were once again single expression cassettes expressing all elements of the regulatable system, the tet-operator and the tet-repressor. Results showed that only the pR20.1/27/TO/TR plasmid was functional in vitro and then only on cells that constitutively expressed the tet repressor (T-REx™-HeLa cells). Before further experimentation in this area it will be necessary to verify that both plasmids express functional tet repressor. In addition the strategy may need to be rethought inasmuch as the system operates best when the tet-repressor to tet-operator are used at a ratio of 6:1 respectively. This could be achieved by using two separate vectors, each one
expressing an element of the system, but the aim here was to create a single vector giving regulatable gene expression.

Collectively the results presented in this thesis have identified replication competent and incompetent HSV vectors capable of gene delivery to spinal ganglia, during both lytic and latent virus infection. These vectors are expected to be extremely useful in the development of gene therapy protocols and/or for the elucidation of gene function in the nervous system. The continuation of development of the regulatable expression vectors, in particular the ecdysone responsive vector is anticipated to provide a means by which gene expression can be precisely controlled and if successful will undoubtedly advance many gene therapy protocols.
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