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**Analysis of the LAT Promoter of
Herpes Simplex Virus Type 1**

by

John Anthony Morrow

**A Thesis Presented For the Degree of Doctor of Philosophy
in the
Faculty of Science, University of Glasgow**

**Institute of Virology,
University of Glasgow**

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SUMMARY

A major feature of the biology of herpes simplex virus type 1 (HSV-1) is its capacity to establish a latent infection in peripheral nervous system ganglionic neurons and to periodically reactivate to produce recrudescence disease.

Until recently the extent and the nature of any expression from latent HSV genomes was unknown. In 1987, latency associated transcripts (LATs) were detected in murine ganglia which were latently infected with HSV-1. The LATs map to the long repeat region and are transcribed in the opposite direction to and partially overlap with IE-1 mRNA. Subsequent work has refined our knowledge of the HSV-1 LATs and shown that transcripts associated with latency are also encoded by a number of other herpesviruses including pseudorabies virus in swine and bovine herpes virus in rabbits.

It is now known that the HSV-1 LATs comprise at least 3 overlapping non-polyadenylated RNAs which share common 5' and 3' ends, but differ in size through differential splicing. These LAT species are thought to be stable introns or stable processing products of a less stable 8.3 kb (m-LAT) polyadenylated primary transcript.

Since LAT appears to be the only actively transcribed gene during latency, the control of LAT gene expression must differ considerably from that of any of the lytic cycle genes. A great deal of attention has thus been focussed on the LAT promoter in order to identify *cis*-acting sequences responsible for this unique pattern of transcription.

In this study, in order to determine the location of the LAT promoter, various sequences upstream from the 2 kb LATs were cloned and examined for their ability to drive expression of an adjoining reporter gene in transient expression assays in a plasmid expression vector. The response of these sequences to the presence of HSV-1 *trans*-

acting factors was also examined by infecting cells transfected with the various LAT promoter/CAT fusion constructs. Both the promoter and HSV-1 responsive elements mapped to a 137 bp region 800 bp upstream from the 5' end of the 2kb LATs. Sequence analysis of this region demonstrated that it contained several homologies to recognised promoter elements including a TATA box, a CAAT box and two putative Sp1 binding sites. Comparison of this region to the corresponding region of the HSV-2 genome revealed that while the 2kb LAT transcription unit itself is completely unconserved, the putative LAT promoter region bears several interesting conserved loci, possibly indicative of the presence of enhancer like elements responsible for tissue-specific expression of the LAT gene.

Several of the LAT promoter/CAT fusion constructs were examined in the context of the virus genome by use of a virus vector whereby DNA sequences of interest are introduced into the viral genome by direct ligation. To facilitate this analysis, the parental vector was modified by deletion of the endogenous LAT sequences, thereby preventing possible rearrangements of the viral genome by homologous recombination between the endogenous and introduced LAT promoter sequences. These recombinant viruses were subsequently used for analysis of the LAT promoter during infection of tissue culture cell lines. The most significant result to come from these studies was the finding that sequences upstream from the core LAT promoter, while not having any effect on LAT promoter strength in BHK cells, considerably enhanced LAT promoter strength in C1300 neuroblastoma cells, thereby suggesting that these sequences harbour neuronal specific enhancer elements.

In addition to studying the LAT promoter, it was considered interesting to examine the behaviour of an endogenous neuronal specific promoter when introduced into the virus genome. To this end, the 5' flanking region of the human neurofilament-light chain gene was obtained, and

two constructs, extending from -296 to +84 and -896 to +84 of the HNF-L promoter fused to the CAT reporter gene were made. Transient transfection assays showed that these constructs performed at least as well as the HSV-1 gD promoter, and were similarly strongly *trans*-activated upon infection of transfected cells with HSV-1. Introduction of the HNF-L/CAT fusions into the HSV-1 genome, and subsequent examination of these constructs during infection of tissue culture cell lines revealed that sequences upstream from -296, while only moderately enhancing promoter activity in BHK cells, had a greater effect in C1300 neuroblastoma cells, thereby suggesting that these sequences perhaps also contained neuronal specific enhancer elements.

The original aim of these studies was to examine promoter/reporter gene activity of the various recombinant viruses in animal latency systems. However, due to lack of time and the necessity of replacing the CAT with the *lacZ* reporter gene for reasons of sensitivity, these studies were unable to be carried out. One virus which was available, (vFJ12) containing the HSV-2 IE-4/5 promoter fused to the *lacZ* reporter gene was used in animal latency studies. *In situ* analysis showed that the parental virus vector expressed LAT in latently infected murine sensory ganglia, thereby demonstrating that this virus could establish latency in this system. Histochemical analysis of dorsal root ganglia infected with vFJ12 showed that during acute infection, a large number of densely stained neurons were observed, indicative of strong IE-promoter activity in those cells. Surprisingly, histochemically stained neurons were also observed in the spinal ganglia of mice after long term latent infection with vFJ12, thereby suggesting that the IE-4/5 promoter may be functional during latency. Genomic analysis of this virus, however showed that some rearrangement had taken place in the U_s region of this virus where the IE-4/5 promoter/*lacZ* fusion had been inserted. While the IE-4/5 promoter was still immediately upstream of the *lacZ* gene, it seemed likely that the deletion resulted

in the *lacZ* gene also coming under control of the LAT transcription unit, and that subsequent long term *lacZ* expression during latency is a consequence of read-through transcription from the LAT promoter.

Nevertheless, the parental 1802/1804 vector should prove to be a useful system for exploring the control of HSV gene expression in neurons, and indeed, may have many interesting applications in neurobiology.

ABBREVIATIONS

| | |
|-----------------|--|
| A | adenine |
| Ac | acetate |
| Amp | ampicillin |
| APS | ammonium persulphate |
| ATP | adenosine-5'-triphosphate |
| BHK | baby hamster kidney cells |
| BHV | bovine herpesvirus |
| BSA | bovine serum albumin |
| C | cytosine |
| ¹⁴ C | carbon-14 radioisotope |
| CAP | chloramphenicol |
| CAV | cell associated virus |
| Ci | curies |
| CIP | calf intestinal phosphatase |
| CMV | cytomegalovirus |
| cpe | cytopathic effect |
| cpm | counts per minute |
| CRV | cell released virus |
| cs | calf serum |
| CTP | cytidine-5'-triphosphate |
| Da | daltons |
| dATP | 2'-deoxyadenosine-5'-triphosphate |
| dCTP | 2'-deoxycytidine-5'-triphosphate |
| dGTP | 2'-deoxyguanosine-5'-triphosphate |
| dTTP | 2'-deoxythymidine-5'-triphosphate |
| dNTP | 2'-deoxyribonucleoside-5'-triphosphate |
| DBP | DNA binding protein |
| del | deletion |
| <i>dl</i> | deletion |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |
| DRG | dorsal root ganglion (ganglia) |
| DTT | dithiothreitol |
| E | early (gene) |

EBV Epstein Barr virus
E. coli *Escherichia coli*
EDTA sodium ethylenediamine tetra-acetic acid
EtBr ethidium bromide
FCS foetal calf serum
G guanine
g grams
gD glycoprotein D
GMEM Glasgow modification of Eagle's medium
h hour(s)
HCMV human cytomegalovirus
HEPES N-2 hydroxyethylpiperazine-N'2 ethansulphonic acid
HGPRT hypoxanthine guanine phosphoribosyltransferase
HHV human herpesvirus
HSV herpes simplex virus
ICP infected cell polypeptide
IE immediate early (gene)
IEC immediate early complex (on TAATGARAT elements)
IF intermediate filament(s)
Ig immunoglobulin
in insertion
IR internal repeat
kb kilobase
l litre
L late (gene)
LAT latency associated transcript
LFP large (klenow) fragment of DNA polymerase
LTR long terminal repeat (promoter)
M molar
min minute(s)
MIR major internal repeat
ml millilitre
mLAT minor latency associated transcript
mM millimolar
moi multiplicity of infection
mRNA messenger ribonucleic acid
M_r molecular weight

MTR morphological transforming region
mu map unit(s)
N unspecified nucleotide or amino acid
n nano
NBC newborn calf serum
NGF nerve growth factor
NPT non-permissive temperature
NP40 Nonidet P40
OD optical density
ORF open reading frame
ori origin of DNA replication
³²P phosphorous 32 radioisotope
pa post-adsorption
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
pfu plaque forming units
pi post infection
poly A polyadenylic acid
PRV pseudorabies virus
R purine moiety
RNA ribonucleic acid
rRNA ribosomal ribonucleic acid
RNase ribonuclease
rpm revolutions per minute
RT room temperature
SDS sodium dodecyl sulphate
sec seconds
syn syncytial plaque forming locus
(*syn*⁺= nonsyncytial, *syn*⁻ = syncytial)
SV40 simian virus 40
T thymidine
TCA trichloroacetic acid
TEMED n,n,n',n'-teramethylethylene diamine
TIF trans inducing factor
TK thymidine kinase
TMPD tetramethylpentadecane
TR terminal repeat

| | |
|----------------|--|
| Tris | tris(hydroxymethyl)aminoethane |
| tRNA | transfer ribonucleic acid |
| ts | temperature sensitive |
| UAS | upstream activating sequences |
| UV | ultraviolet |
| V | volt |
| V _m | apparent molecular weight of virus-induced protein |
| vol | volume(s) |
| v/v | volume/volume |
| VZV | varicella zoster virus |
| wt | wild type |
| w/v | weight/volume |
| Y | pyrimidine moiety |
| X-gal | 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside |

Greek symbols

| | |
|---|---------|
| α | alpha |
| β | beta |
| γ | gamma |
| μ | micro |
| ε | epsilon |

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CHAPTER 1: INTRODUCTION

1A. BIOLOGY OF THE HERPESVIRIDAE

1A.1. Structure and Classification

The Herpesviridae are a morphologically homogeneous family of large DNA-containing animal viruses. Herpesvirus particles characteristically consist of an icosahedral nucleocapsid containing a linear double-stranded DNA genome of between 0.1 and 0.25 Mb in size.

Electron-microscopic analysis has revealed that the herpes virion consists of four distinct morphological features:

i) an electron-dense core, toroidal in conformation and consisting of a DNA/protein complex (Epstein, 1962b, Zamberland & Vatter, 1966, Furlong *et al.*, 1972, Nazerian, 1974).

ii) an icosahedral capsid, 125 nm in diameter and comprising 12 pentameric capsomeres and 150 hexameric capsomeres, each being 12 nm in diameter (Wildy *et al.*, 1960, Schrag *et al.*, 1989, Baker *et al.*, 1990).

iii) a tegument, consisting of a largely undefined amorphous proteinaceous layer between the capsid and envelope (Roizman & Furlong, 1974, Epstein, 1962b, Spear, 1980).

iv) an envelope of typical trilaminar appearance which surrounds the capsid and tegument, and is derived by budding through the inner nuclear membrane (Epstein 1962a, Nii *et al.*, 1968, Asher *et al.*, 1969). The envelope contains numerous virally encoded glycoprotein spikes,

approximately 8 nm in length (Wildy & Watson, 1963, Spear & Roizman, 1972).

Herpesvirus classification into three main subgroups- α , β , and γ - has been based largely on host range and tropism, as well as *in vitro* growth rate and characteristics of viral latency (Roizman, 1982). Each of these groups embrace a large number of viruses, (Hones, 1984), but here, specific reference will only be made to the stereotypic human herpesviruses which happen to be the best studied of the herpesviridae.

1A.2. Human Herpesviruses and Disease

There are at least 6 known herpesviruses whose natural host is man, and these include representatives of all 3 subfamilies:

2.1. The α -Herpesviruses: These 'neurotropic' viruses typically cause mild primary infections of epithelial cells of skin giving rise to vesicular lesions. HSV-1 is typically associated with lesions of the eyes (herpes keratitis) and oral cavity (herpes labialis), while HSV-2 is predominantly associated with lesions of the genital tract (herpes genitalis), (Whitely, 1985). Following primary infection, alphaherpesviruses typically persist in a latent form and may give rise to periodic recurrences of mild disease. The normal sites of latency appear to be the ganglionic neurons innervating the primarily infected area (Baringer & Swoveland, 1973, Fraser *et al.*, 1981). HSV has also been associated with serious generalized infections of neonates, or a rare, but fatal encephalitis in adults (Whitely, 1985). Spread of the virus is typically by oral or genital contact.

Primary infection of varicella zoster virus (VZV) results in the childhood disease known as chicken pox

(varicella). VZV can establish a latent infection in dorsal root ganglia during primary infection. Upon reactivation, the cutaneous disease herpes zoster (shingles), a predominantly adult disease may be produced. *In vitro*, alphaherpesviruses typically have a broad host range and relatively rapid growth cycle (<18h).

2.2. The β -Herpesviruses: Members of this 'salivary-gland' virus group tend to be *in vitro* species-specific. A betaherpesvirus prototype is human cytomegalovirus (HCMV). HCMV infection in normal adults is widespread, but results in a benign and asymptomatic disease. However, disease can be disseminated and severe in unborn children or in immunosuppressed patients, particularly in transplant therapy, and more recently, in patients with acquired immune deficiency syndrome (AIDS). The virus can be passed from mother to foetus and is a major cause of congenital damage in infants. The site(s) and essential features of betaherpesvirus latency are unknown, but they can be isolated from salivary gland epithelium, lymphoreticular cells and kidney cells. The reproduction cycle is slow and is characterized by cell enlargement (cytomeg^olia) and presence of DNA inclusions in the nucleus.

2.3. The γ -Herpesviruses: These 'lymphotrophic' viruses are typified by the B-cell lymphotropic Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis (glandular fever). In African populations, EBV is associated with Burkitts lymphoma (a B-cell malignancy), and in Asian populations, with nasopharyngeal carcinoma (an epithelial cell malignancy). Gammaherpesvirus *in vitro* host range is usually restricted to the same family or order as the natural host. EBV is able to establish latency in lymphocytes. It efficiently immortalizes B-lymphocytes *in vitro* which has facilitated the study of a model gammaherpesvirus latent state (Dambaugh *et al.*, 1986,

Epstein & Achong, 1986).

A sixth human herpesvirus was described in 1986. It was first termed human B-lymphotropic virus (Josephs *et al.*, 1986, Salahuddin *et al.*, 1986) but is now known as human herpesvirus-6 (HHV-6) (Ablashi *et al.*, 1987). It was first isolated from patients with AIDS or lymphoproliferative disorders, however, the only disease in which HHV-6 has been implicated is exanthem subitum, a transient childhood illness (Yamanishi *et al.*, 1988). It is now thought that this virus can grow in lymphocytes of both B and T lineages (Downing *et al.*, 1987, Lopez *et al.*, 1988). Classification of this virus has proven to be problematic; although originally classified in the gamma subfamily on the basis of its ability to infect lymphoid cell lines, sequencing studies show that at the molecular level it is more closely related to HCMV (Lawrence *et al.*, 1990).

The isolation of a seventh human herpesvirus from CD4⁺ T cells has recently been reported (Frenkel *et al.*, 1990). This virus, termed human herpesvirus 7 (HHV-7), appears to be partially related to HHV-6 as identified by restriction enzyme and blot hybridization analysis. No such cross-hybridization was observed with probes from HSV, EBV or HCMV.

1A.3. Transformation and Oncogenesis

HSV and HCMV have long been associated with the induction of premalignant or malignant disease; particularly HSV-2 in the case of squamous cell carcinoma of the cervix. Early links between HSV and neoplastic disease were based on seroepidemiological studies (reviewed by Rawls, 1983; Vonka *et al.*, 1984). However, any direct

role for HSV-2 in the development of cervical carcinoma has proven difficult to evaluate; the presence of viral DNA sequences has only been detected in a small proportion (about 10%) of cervical carcinoma tissues, in contrast to human papilloma virus (HPV), which is strongly associated with human genital neoplasia, being present in over 80% of tumours (reviewed by Macnab, 1987).

Morphological studies have failed to demonstrate HSV encoded transforming genes (Cameron *et al.*, 1985) similar to those of the DNA tumour viruses eg, adeno and papova viruses. However, 3 regions of the HSV genome and one region from the HCMV genome (termed morphological transforming regions; MTRs) have been demonstrated to induce transformation of cultured cells. These regions are: MTR I, *XbaI f*, (0.29 to 0.45 m.u.) and *BglIII i*, (0.311 to 0.415 mu) of HSV-1, (Reyes *et al.*, 1979); MTR II, mapping in HSV-2 *BglIII n*, (0.58 to 0.62 mu), (Reyes *et al.*, 1979; Macnab & McDougall, 1980; Galloway & McDougall, 1981; Cameron *et al.*, 1985); MTR III, mapping in HSV-2 *BglIII c*, (0.54 to 0.58 mu), (Jariwalla *et al.*, 1983) and HCMV MTR *HindIII E* (0.123 to 0.140), (Galloway *et al.*, 1984; Nelson *et al.*, 1984). These transforming regions share no homologies with each other. Moreover, surprisingly small subfragments of these regions, unable to encode any intact proteins, have been shown to induce morphological transformation (Galloway *et al.*, 1984). Also, the DNA used to induce morphological transformation is not necessarily maintained within the transformed cell (Galloway & McDougall, 1983; Galloway *et al.*, 1984; Cameron *et al.*, 1985; Jariwalla *et al.*, 1986), which possibly suggests some kind of "hit and run" mechanism (Skinner, 1976; Minson *et al.*, 1976), where the DNA is involved in the initiation of transformation, yet its presence is not required for maintenance of the transformed state.

One model has been suggested where the specific arrangement of DNA in these sequences may initiate

transformation; Galloway *et al.*, (1984) proposed that small stem/loop structures of 737 bp in MTR II and 490 bp in the HCMV MTR may induce morphological transformation, acting either as a mutagen or transcriptional activator. However, there is no evidence that these structures have any significance, indeed, it is possible to generate similar structures with randomly shuffled DNA sequences (Macnab, 1987).

There are many possible mechanisms whereby HSV may disrupt normal cellular gene regulation or perhaps even cause rearrangements or gene amplification. HSV has been shown to act as a mutagen on a cellular reporter gene (Pilon *et al.*, 1985; 1986) and also induce chromosome damage (Peat & Stanley, 1986) and gene amplification (Lavi, 1981). HSV has the potential to elevate the expression of various cellular genes upon infection, (LaThangue *et al.*, 1984; Kennedy *et al.*, 1985; Macnab *et al.*, 1985; Patel *et al.*, 1986; Latchman *et al.*, 1987), occurring directly through the HSV transcriptional activators or as a result of the mutagenic activity of HSV gene products involved in DNA metabolism and rearrangement. Indeed, some HSV-1 transformed cell lines have been shown to transcribe certain cellular genes at elevated levels (Filion *et al.*, 1988) and some cellular polypeptides expressed at increased levels in infected cells also accumulate to higher levels in transformed cells (Macnab, 1985).

1A.4. Genome Structures of the Human Herpesviruses

The structure and organisation of herpesvirus genomes has aroused great interest since it was demonstrated that HSV-1 DNA could be isolated from virions as a linear duplex molecule containing single-stranded nicks (Kieff *et al.*, 1971; Wilkie, 1973).

The HSV-1 DNA is regarded as consisting of two covalently linked segments termed the Long (L) and short (S) regions. Each of these contains a unique sequence, (U_L and U_S) which is flanked by a pair of oppositely oriented repeat sequences (R_L and R_S). The terminal and internal copies of R_L are termed TR_L and IR_L respectively; for R_S , these are termed TR_S and IR_S . (see Figure 1; Sheldrick and Berthelot, 1974). The molecules also possess a terminal redundancy of some 400 bp, termed the α sequence; one or more additional copies of this sequence are located internally at the joint region between the L and S segments in opposite orientation to the terminal α sequences (Sheldrick & Berthelot, 1974; Wadsworth *et al.*, 1975, Wagner & Summers, 1978; McGeoch *et al.*, 1986). Each terminus possesses one overhanging 3' residue, with the 3' hydroxyl group free (Mocarski & Roizman, 1982a). In a productively infected cell, an inversion process occurs at the internal α sequence so that the progeny virion DNA consists of a mixture of four sequence-oriented isomers which differ in the relative orientations of their L and S segments, (Hayward *et al.*, 1975; Delius & Clements, 1976; Wilkie, 1976). One isomer is designated the prototype for mapping purposes (Roizman, 1979).

The full genomic sequence of HSV-1 strain 17+ became available in 1988 and was shown to comprise 152,260 residues in each strand (McGeoch *et al.*, 1985; McGeoch *et al.*, 1986; McGeoch *et al.*, 1988a; Perry & McGeoch, 1988), however this value will probably vary somewhat due to the natural variability of different HSV-1 strains and different copy numbers of reiterations. HSV-1 DNA has a base composition of 68.3% G+C (McGeoch *et al.*, 1988a) although the actual composition of specific regions of the genome may deviate from this value, eg, the 6.6 kbp R_S with a G+C content of 79.5%. The genome of HSV-1 is predicted as possessing 74 genes, densely arranged, and encoding 71 distinct proteins. There are 12 genes in U_S and 56 in U_L ,

Figure 1. Gross structures of the human herpesviruses. Each linear genomic DNA is shown with unique sequences as heavy lines and repeat elements as boxes with relative orientations indicated by arrows.

Nomenclature:

| | |
|-------------------|---------------------------------|
| U _L | Unique-long |
| U _S | Unique-short |
| I/TR _L | Internal/terminal long repeat. |
| I/TR _S | Internal/terminal short repeat. |
| MIR | Major internal repeat. |
| D _R | Direct repeat (right). |
| D _L | Direct repeat (left). |

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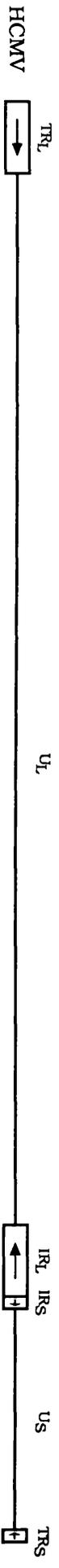


Figure 2. Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORFs are shown by arrows. Overlaps of adjacent, similarly oriented ORFs are not shown explicitly. Locations of proposed transcription polyadenylation sites are shown as short vertical bars. Locations of origins of DNA replication are indicated as X. In the U_L region, on the first three lines, genes UL1 to UL56 are labelled. In the U_S region, on the bottom line, genes US1 to US12 are labelled. The locations of introns in the coding regions of gene UL15 and the two copies of the IE110 gene are indicated. Three additional genes are not shown. These are ICP34.5 (see Figure 5), UL26.5, and UL49.5.

This Figure is taken directly from McGeoch *et al.* (1988a) with permission.

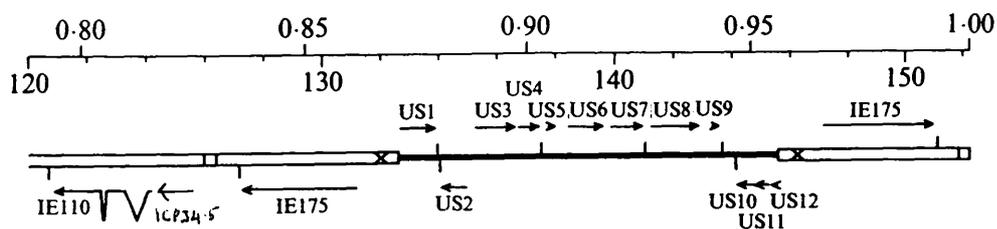
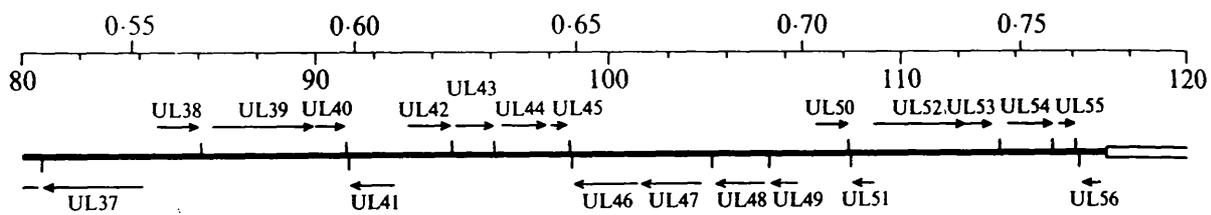
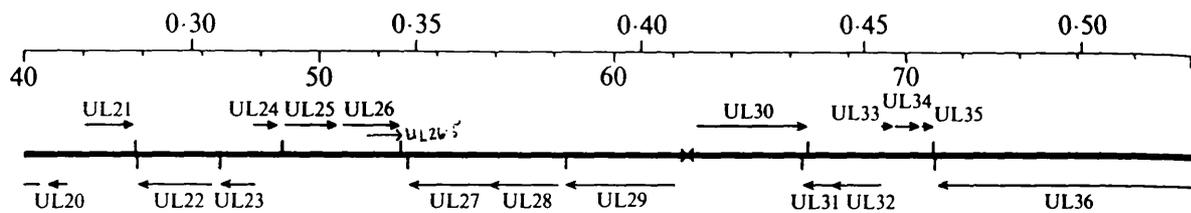
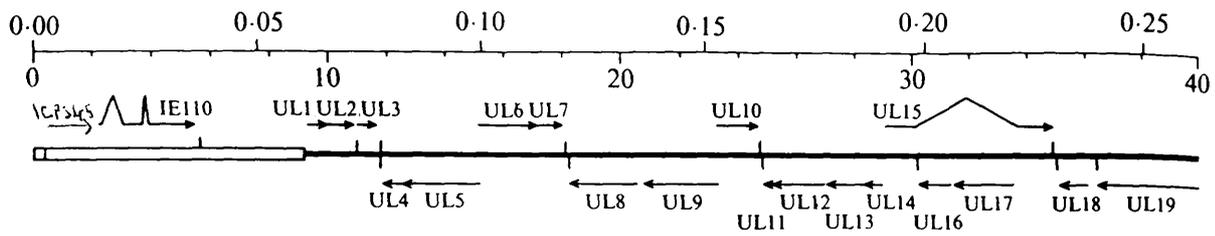


Table 1. Summary of functions and phenotypes for genes of HSV-1.

| <u>HSV-1 gene</u> | <u>Protein and/or function</u> |
|-------------------|---|
| ICP34.5 | Neurovirulence |
| IE110 | IE transcriptional regulatory protein |
| UL1 | Unknown |
| UL2 | Uracil-DNA glycosylase |
| UL3 | Unknown |
| UL4 | Unknown |
| UL5 | Required for DNA replication; probable DNA helicase component |
| UL6 | Presumed virion protein; possible role in DNA packaging |
| UL7 | Unknown |
| UL8 | Required for DNA replication; function unknown |
| UL9 | Required for DNA replication; ori-binding protein |
| UL10 | Function unknown; possible membrane inserted protein |
| UL11 | Unknown |
| UL12 | Deoxyribonuclease |
| UL13 | Predicted protein kinase |
| UL14 | Unknown |
| UL15 | |
| exon 1 | Unknown (related to T4 packaging protein) |
| exon 2 | |
| UL16 | Unknown |
| UL17 | Unknown |
| UL18 | Capsid protein |
| UL19 | Major capsid protein |
| UL20 | Unknown |
| UL21 | Unknown |
| UL22 | Glycoprotein H |
| UL23 | Thymidine kinase |
| UL24 | Unknown |
| UL25 | Virion protein |
| UL26 | Protease |
| UL26.5 | Capsid assembly protein |
| UL27 | Glycoprotein B |
| UL28 | Probable structural protein |
| UL29 | ssDNA binding protein required for DNA replication |
| UL30 | Replicative DNA polymerase |
| UL31 | Unknown |
| UL32 | Probable structural protein |
| UL33 | Structural; involved in DNA packaging |
| UL34 | Possible virion protein |
| UL35 | Capsid protein |
| UL36 | Very large tegument protein |
| UL37 | Unknown |
| UL38 | Capsid protein |

| | |
|--------|--|
| UL39 | Ribonucleotide reductase large subunit |
| UL40 | Ribonucleotide reductase small subunit |
| UL41 | Presumed virion protein; virion host shutoff |
| UL42 | DNA binding protein, required for DNA replication |
| UL43 | Unknown |
| UL44 | Glycoprotein C |
| UL45 | Virion protein |
| UL46 | Unknown |
| UL47 | Unknown. May modulate activity of UL48 protein |
| UL48 | Major tegument protein, <i>transactivates</i> IE genes |
| UL49 | Tegument protein |
| UL49.5 | Possible membrane protein |
| UL50 | Deoxyuridine triphosphatase |
| UL51 | Unknown |
| UL52 | Required for DNA replication, function unknown |
| UL53 | Possible membrane protein |
| UL54 | IE transcriptional regulatory protein |
| UL55 | Unknown |
| UL56 | Unknown |
| IE175 | IE transcriptional regulatory protein |
| US1 | IE protein |
| US2 | Unknown function |
| US3 | Protein Kinase |
| US4 | Glycoprotein G |
| US5 | Glycoprotein J |
| US6 | Glycoprotein D |
| US7 | Glycoprotein I |
| US8 | Glycoprotein E |
| US9 | Virion protein |
| US10 | Virion protein |
| US11 | Function unknown; localizes in nucleolus |
| US12 | IE protein |

This table is adapted from an original table published in McGeoch, (1989), with permission.

while R_L contains 2 genes encoding Vmw110 and ICP34.5, and R_S contains 1 gene encoding Vmw175. The genomic location of the HSV-1 genes are shown on Figure 2, and a summary of their functions are given on Table 1.

The genomic structure of HSV-2, though not completely sequenced yet, is essentially similar to that of HSV-1, but with a slightly higher G+C content. DNA hybridization studies have demonstrated that the HSV-1 and HSV-2 genome sequences are highly related and closely colinear (Kieff *et al.*, 1972; Davison & Wilkie, 1983). This has been borne out by the demonstration that intertypic recombinants between HSV-1 and HSV-2 are viable (Wilkie *et al.*, 1977). It therefore seems very likely that the genetic organization of HSV-2 will be found to be nearly indistinguishable from HSV-1. Coding sequences of corresponding genes show 70%-80% identities (McGeoch *et al.*, 1987) with homology being less pronounced in non-coding regions, and most divergence occurring in parts of the major repeats (Davison & Wilkie, 1981; Whitton & Clements, 1984).

The genome of VZV, also a double-stranded DNA molecule, has been demonstrated by DNA sequencing to consist of 124,884 bp with a base composition of 46.0% G+C (Davison, 1983; Davison & Scott, 1983). The genome consists of two covalently-linked regions, L and S, thus resembling that of HSV-1, except that where U_S is flanked by large inverted repeats (TR_S , IR_S ; 7320 bp), U_L is flanked by inverted repeats of only 88 bp. VZV does not possess a terminal redundancy, and as with HSV-1, each terminus has an unpaired residue (Davison, 1984). VZV DNA also occurs in four sequence-oriented isomers, however, two of the isomers (representing one orientation of U_L), are about 20-fold more abundant than the other two (Davison, 1984).

The EBV genome, the first herpesvirus genome to be sequenced, is a linear double-stranded molecule of prototype sequence 172,282 bp with 59.9% G+C content (Baer

et al., 1984). However, further analysis indicated that the prototype strain, B95-8, was an unusual deletion derivative (an artifact common in laboratory strains maintained in cell lines), (Raab-Traub *et al.*, 1980; Laux *et al.*, 1985), indicating that the true length of the EBV genome is probably closer to 186 kbp. The EBV genome termini consist of tandem repeats of 538 bp (termed terminal repeats; TR;) (Given *et al.* 1979; Kintner *et al.*, 1979). A variable number of large internal repeats of 3072 bp, termed major internal repeats (MIRs), join a short and long unique region (Given & Kieff, 1979; Rymo & Forsblom, 1978; Hayward *et al.* 1980). Another set of two 1000bp repeat regions of nearly identical sequence, termed D_L and D_R, are located near the extremities of U_L (Laux *et al.*, 1985; Raab-Traub *et al.*, 1980). These elements lie in the same orientation, and each is adjacent to a family of tandemly reiterated sequences.

The HCMV genome is structurally similar to HSV with respect to the layout of unique and repeat sequences, (Geelen *et al.*, 1978; Kilpatrick & Huang, 1977; Stinski *et al.*, 1979; Weststrate *et al.*, 1983). It possesses the equivalent of an α sequence (Spaete & Mocarski, 1985) and also occurs in four sequence-oriented isomers. HCMV, at 230 kbp with a base composition of 56% G+C, is the largest of the human herpesvirus genomes (Chee *et al.*, 1990)

Information concerning the structure of the HHV-6 genome is scarce, however, it is thought to contain around 160 kbp (42% G+C) in a structure consisting of a single unique sequence flanked by a pair of large direct repeats (Lawrence *et al.*, 1990). As yet, no information at all exists concerning the genomic structure/content of the putative HHV-7.

1A.5. THE HSV-1 LYTIC CYCLE

5.1. Adsorption and Penetration

Virus entry into a cell requires virus attachment and penetration of the cell surface (Morgan *et al.*, 1968). Adsorption and penetration are distinct processes involving different components of the virion envelope. Most work concerning the virion components has centred upon the viral glycoproteins gB, gD and gH which have been shown to be indispensable for virion infectivity (Sarmiento *et al.*, 1979; Cai *et al.*, 1987; Desai *et al.*, 1988; Ligas & Johnson, 1988; Little *et al.*, 1981). HSV virions contain at least five other membrane glycoproteins, (gC, gG, gI, gE and gJ), however, these seem to be dispensible for infection of cultured cells, (Heine *et al.*, 1974; Homa *et al.*, 1986a; Longnecker & Roizman, 1987; Neidhardt *et al.*, 1987).

The initial attachment of HSV to cells involves the binding of the virion to heparan sulphate moieties of cell surface proteoglycans (WuDunn & Spear 1989). Neither gB nor gD are involved in this initial attachment as virions devoid of either glycoprotein can adsorb to cells normally (Cai *et al.*, 1988; Ligas & Johnson, 1988). Although not tested yet, it seems likely that gH-negative mutants will also prove to be adsorption competent and penetration negative. The finding that monoclonal antibodies specific for gB (Highlander *et al.*, 1988), gD (Fuller *et al.*, 1987; Highlander *et al.*, 1987) and gH (Fuller *et al.*, 1989), and *ts* mutants of these proteins; gB (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Cai *et al.*, 1988) and gD (Ligas & Johnson, 1988, Johnson & Ligas, 1988), can block penetration without blocking adsorption are consistent with the role for these proteins in steps subsequent to initial adsorption. In addition there is evidence that the UL25 gene product, a probable tegument protein, is required at a

late stage of viral entry since the mutant *ts1204* which has a lesion in this polypeptide has a penetration defect at the NPT (Addison *et al.*, 1984).

Although the initial binding of HSV to cells requires the presence of heparan sulphate on the cell surface, stable attachment may require subsequent interactions of the virion glycoproteins with other cell surface receptors. Indeed, there is evidence that gD interacts with its own distinct cell surface receptor (Fuller & Spear, 1985; Campadelli-Fiume *et al.*, 1988; Johnson & Ligas, 1988; Ligas & Johnson *et al.*, 1988; Johnson & Spear, 1989). It is still not clear whether gB and gH interact with specific cell surface receptors.

Once fusion of the viral envelope and cell membrane has taken place, the viral nucleocapsid and tegument proteins are released into the cytoplasm (Morgan *et al.*, 1968). Once inside the cell, nucleocapsids accumulate at nuclear pores where the viral DNA is released; a *ts* mutant mapping to UL36, a very large tegument protein of unknown function is deficient in releasing virion DNA from capsids at the nuclear pores (Batterson *et al.*, 1983; Knipe *et al.*, 1981).

5.2. The Effect of HSV on Host Cell Macromolecular Synthesis

Infection of tissue culture cells by HSV causes a well characterized decline in host cell macromolecular synthesis that typically occurs within 2-4 hours post-infection in a strain dependent manner (reviewed by Fenwick, 1984). Early studies indicated that upon HSV infection, synthesis of cellular DNA is blocked (Roizman & Roane, 1964), and that cell mitosis is arrested (Wildy *et al.*, 1961). HSV-1 infection has also been shown to induce specific chromosome damage at early times of infection,

which becomes more extensive at late times (Peat & Stanley, 1986).

Inhibition of host protein synthesis has been clearly shown to be brought about by the action of a component of the HSV virus particle; the product of gene UL41, termed the virion host shutoff (*vhs*) function (Nishioka & Silverstein, 1978; Fenwick & Walker, 1978; Kwong *et al.*, 1988; Fenwick & Everett, 1990; Smibert *et al.*, 1992). The consequence of UL41 gene function is the shut-off of cellular protein synthesis which is mediated at a number of levels including disaggregation of polyribosomes synthesising cellular proteins (Sydiskis & Roizman, 1966, 1967; Nishioka & Silverstein, 1978) and the degradation of cellular mRNAs (Nishioka & Silverstein, 1977, 1978; Schek & Bachenheimer, 1985). Inactivation of the *vhs* gene has also been associated with increased stability of viral mRNAs (Read & Frenkel, 1983; Oroskar & Read, 1987, 1989; Kwong & Frenkel, 1987; Kwong *et al.*, 1988), indicating that RNA degradation probably occurs by an indiscriminate mechanism. Mitochondrial RNAs have also been shown to be degraded upon HSV infection (Latchman, 1988). In addition to *vhs*, it also appears that a 'late' shutoff function requiring expression of early and late polypeptides is required for full and efficient shutoff of host polypeptide synthesis (Nishioka & Silverstein, 1978; Read & Frenkel, 1983).

The efficiency of *vhs* differs between different strains of HSV; HSV-2 strain G displays a strong shutoff phenotype, whereas HSV-1 strain 17 syn⁺ is weak and HSV-2 strain HG52 is completely deficient (Pereira *et al.*, 1977; Schek & Bachenheimer; 1985 Fenwick & Owen, 1988). The latter can be explained by a frameshift mutation within the HSV-2 UL41 coding sequence and thus synthesizes a truncated UL41 gene product (Everett & Fenwick, 1990). Surprisingly, in mixed infection experiments using weak and strong shutoff viruses, the weak UL41 allele generally inhibits

the shutoff induced by the stronger one. (Hill *et al.*, 1985; Fenwick & Everett, 1990). Host shutoff therefore probably results in preferential synthesis of viral proteins due to the more rapid synthesis of their messages; *vhs* RNA degradation probably facilitates the rapid transitions in expression of the temporally regulated groups of viral genes that are transcriptionally active at different times post-infection (Kwong *et al.*, 1988). The UL41 gene product has recently been identified by the use of rabbit antipeptide antiserum as a 58K phosphoprotein in infected cells (Smibert *et al.*, 1992).

Another effect of HSV on host macromolecular synthesis is that, despite *vhs*, a small number of cellular proteins are observed to increase in abundance following infection. Activation of cellular stress protein gene expression occurs after infection with HSV-1 *ts* mutants which overproduce IE polypeptides at NPT (Notarianni & Preston, 1982; Russell *et al.*, 1987a). Other studies have indicated that infection with HSV-2 induces synthesis of minor cellular stress proteins (LaThangue, *et al.*, 1984; Patel *et al.*, 1986; Kennedy *et al.*, 1986), and that their synthesis is stably increased in cells transformed by HSV (Macnab *et al.*, 1985). In some cases, cellular gene induction has been shown to be dependent on the expression of the viral immediate-early transactivating protein Vmw175 (Latchman *et al.*, 1987; Kemp & Latchman, 1988). In other cases it is independent of viral protein synthesis (Kemp *et al.*, 1986). Cellular promoters introduced into cell lines by biochemical transformation can also be activated by HSV-1 infection, and this involves the action of IE transactivating proteins, Vmw110 and Vmw175 (Everett, 1985). In addition, a recent report has also demonstrated that binding of the HSV virion to the cell surface directly induces a, so far uncharacterized, 56,000 molecular weight protein (Preston, 1990).

5.3. HSV DNA REPLICATION

Studies of DNA replication have been hampered by the large size of the HSV genome. However, analysis of replication intermediates using pulsed labelling and electron microscopy have shown that DNA pulse-labelled *in vivo* sediments more rapidly than unit length DNA (Jacob & Roizman, 1977), and that it is "endless" ie, the molecular termini are fused together (Jacob *et al.*, 1979; Jongeneel & Bachenheimer, 1981). On this basis it is proposed that parental viral DNA is circularized shortly after entry into the host cell, and that replication takes place by a rolling circle mechanism generating linear concatamers of tandemly repeated viral genomes (Jacob & Roizman, 1979). This hypothesis is largely untested and other mechanisms eg the 'Cairns' type mechanism (which may also account for higher sedimentation) cannot be discounted (Jongeneel & Bachenheimer, 1981).

3a. The Replication Origins

HSV contains two distinct origin sequences (Vlazny & Frenkel, 1981). Both have been cloned from the wild-type viral genome (Stow, 1982; Stow & McMonagle, 1983; Weller *et al.*, 1985) and plasmids containing either of these sequences have been shown to be amplified when cells transfected with them are infected with HSV. *Ori_S* is located within the inverted repeat of *U_S* and thus is present in two copies (Mocarski & Roizman, 1982b; Stow, 1982; Stow & McMonagle, 1983). *Ori_L* is located in the middle of *U_L* (Locker *et al.*, 1982). *Ori_S* appears to consist of less than 100 bp, including a 45-bp dyad symmetry with a central region of 18-bp containing only A and T residues; *ori_L* is very similar, but the dyad symmetrical region is longer (Murchie & McGeoch, 1982; Knopf *et al.*, 1986). The functional significance of three separate origins of

replication is not clear. Mutants lacking *ori_L* or one *oris* appear to be viable (Longnecker & Roizman, 1986; Polvino-Bodnar *et al.*, 1987). However, it has proven impossible to construct a virus lacking both *oris* sequences hence implying that virus replication requires at least two origin sequences whether they be *ori_L* or *oris*. It has been demonstrated that two arms of the *oris* palindrome serve as UL9 binding sites (see below) and the central AT-rich region has been proposed as a potential site of protein-induced unwinding. However, many questions remain regarding the HSV origins. It seems likely that DNA replication begins at one or more of the origin sequences however, as yet, no information exists on the molecular events by which HSV DNA replication is initiated.

3b. Viral Genes Required for DNA Replication

The complete set of viral genes required for DNA replication were identified by means of a transient complementation assay in which cloned HSV-1 DNA fragments were tested for the ability to support replication of a cotransfected plasmid containing *oris* or *ori_L* (Challberg, 1986). Seven genes were found to be both necessary and sufficient for origin-dependent DNA synthesis (Wu *et al.*, 1988). The functions of the products of most of the genes are either unknown or only recently identified. These are listed below:

DNA Polymerase (UL30). Most highly purified preparations consist predominantly of a monomer of single polypeptide chain about 140 kd in size (O'Donnell *et al.*, 1987; Powell & Purifoy, 1977) in good agreement with the size of product as predicted from the DNA sequence (Wu *et al.*, 1988). The polypeptide is in itself a catalytically active polymerase, however it is not clear whether accessory factors may be required to increase efficiency or modify activity in some

UL42 is proposed to serve as a processivity factor for viral DNA polymerase, and that a heterodimer of UL30 and UL42 represents the functional DNA polymerase holoenzyme (Gottlieb *et al.*, 1990).

way. As with many other polymerases, it has an intrinsic 3'-5' exonuclease activity that probably serves as a proofreading function to increase fidelity of DNA synthesis (Haffey *et al.*, 1988).

UL42. A DNA binding protein of as yet unknown function (Parris *et al.*, 1988). In HSV-2, it can be co-purified with DNA polymerase as a 55 kd protein (Powell & Purifoy, 1977). Its effect, if any, on the DNA polymerase has yet to be determined.

Single-Stranded DNA Binding Protein (UL29). Recognized as an abundant HSV-1 induced protein of 130 kd present in infected cells (Hones & Roizman, 1973; Powell & Courtney, 1975) and has been shown to bind tightly to single-stranded DNA cellulose columns (Ruyechan, 1983). Its function is presumably to bind single-stranded DNA present at the replication fork formed by unwinding of the parental duplex, and to facilitate the use of these strands as templates for DNA polymerase.

Origin-Binding Protein (UL9). Originally identified in HSV infected cell extracts (Elias *et al.*, 1986). Consists of a single major polypeptide of about 83 kd (McGeoch *et al.*, 1988b). UL9 protein has been expressed using a baculovirus expression system and demonstrated to interact with two nearly identical sites located on each arm of the *oris* palindrome (Olivo *et al.*, 1988; Weir *et al.*, 1989; Weir & Stow, 1990). The role of UL9 binding in HSV DNA replication is not clear, however by analogy with other prokaryotic and eukaryotic origin recognition proteins, the binding of UL9 to *oris* or *oriL* may initiate assembly of a multiprotein replication complex. It has recently been shown, however, that the ability of the UL9 protein to bind to the viral origins of replication is not sufficient for it to facilitate DNA synthesis (Stow, 1992). It is also possible

that it is involved in unwinding the two parental strands at the origin before DNA synthesis.

Helicase/Primase. Infection of cells with HSV induces novel helicase and primase activities (Crute *et al.*, 1988; Holmes *et al.*, 1988). Both activities are components of a 3-subunit enzyme composed of the products of the UL5, UL8 and UL52 genes (Crute *et al.*, 1989). The helicase can utilize either ATP or GTP as a co-factor for unwinding and moves in a 3'-5' direction on the strand to which it is bound (Hodgman, 1988; Zhu & Weller, 1988). This suggests that the enzyme may prime lagging strand synthesis as it unwinds DNA at the replication fork. The activities of the component polypeptides at the complex have yet to be determined, although UL5 contains several sequence motifs that are shared by helicases (McGeoch *et al.*, 1988b).

Although HSV-1 contains only seven genes that are required for viral DNA replication in cultured cells, the virus encodes several other proteins likely to be involved in nucleoside/nucleotide metabolism, and DNA repair. These include a thymidine kinase (UL23)(Kit & Dubbs, 1963), a ribonucleotide reductase (UL39 and UL40)(Frame *et al.*, 1985; Preston *et al.*, 1984), a dUTPase (UL50)(Fisher & Preston, 1986), a uracil-DNA glycosylase (UL2)(Worrad & Carradonna, 1988; Mullaney *et al.*, 1989) and an alkaline exonuclease (UL12)(Preston & Cordingly, 1982), although a convincing role has not been identified for the latter. Genetic studies have demonstrated that while mutations in these genes have only minor or no effects on viral DNA synthesis in infected tissue culture cells, they may cause profound defects in the ability of the virus to replicate following experimental infection of animals (Cameron *et al.*, 1988; Goldstein & Weller, 1988a). It is likely that rapidly dividing cultured cells may provide functions that are lacking in non-mitotic cells encountered by the virus

in a natural infection. Whether host cells provide functions necessary for viral DNA replication that are not virus-encoded remains to be determined.

Despite the large body of information now accumulating concerning HSV DNA replication, any further in-depth analysis of this process requires the development of a soluble origin-dependent *in vitro* system.

5.4. Virus Maturation.

Following replication, the large concatameric HSV genomes are cleaved within the *a* sequences situated at the junctions between adjacent viral genomes. Studies with temperature-sensitive mutants have suggested that the cleavage process may be linked to packaging (Ladin *et al.*, 1982; Preston *et al.*, 1983), with the *a* sequences probably serving as the recognition sequence for the machinery that processes concatamers to unit length during the encapsidation process (Stow *et al.*, 1983; Varmuza & Smiley, 1985; Diess *et al.*, 1986). The product of gene UL26, termed p40, has been shown to be present in empty capsids, but not present as a component of full capsids or mature virions indicating that it is transiently associated with capsids, and that its removal from capsids is linked with the process of DNA packaging (Rixon *et al.*, 1988; Sherman & Bachenheimer, 1988). Any pair of *a* sequences along the concatameric molecule can be cleaved resulting in the packaging of monomers, dimers and higher multimeric forms into nuclear capsids. However, the multimeric forms do not contain any intact genomes, and only nucleocapsids containing unit length DNA are enveloped (Vlazny *et al.*, 1982).

The classical theory of final virion maturation is that intranuclear capsids become enveloped by budding through the inner lamella of the nuclear membrane (which

has been modified by viral glycoproteins) into the perinuclear space (Roizman & Furlong, 1974). Virions are then transported to the cytoplasmic membrane, possibly via the golgi apparatus, where virus egress typically initiates at around 8 hrs post-infection (Johnson & Spear, 1982). Recent studies, however, using the β -herpesvirus HHV-6 (Roffman *et al.*, 1990) and the α -herpesviruses PRV (Whealy *et al.*, 1991) and HSV-1 (Cheung *et al.*, 1991) support the belief that envelopment of nascent capsids takes place in the cytoplasm. In their model, Roffman *et al.* (1990) suggest that the envelope gained by the intranuclear capsids by budding through the inner nuclear membrane is lost by subsequent fusion with the outer nuclear membrane, culminating in release of the capsid into the cytoplasm. They also suggest that prior to this, tegumentation of the HHV-6 capsid occurs in a cytoplasmic inclusion in the nucleus termed the tegusome. Evidence for tegusome-like compartments has not been found in HSV-1 infected cells (personal communication, F.J. Rixon), and tegumentation of HSV-1 therefore probably occurs in the cytoplasm prior to envelopment by budding into the Golgi apparatus.

1B. HSV-1 GENE REGULATION

1B.1. An Overview

The expression of HSV-1 genes during infection of tissue culture cells can be divided into at least 3 temporally regulated classes termed immediate-early (IE or alpha), early (E or beta) and late (L or gamma) (Hones & Roizman, 1974; Clements *et al.*, 1977). Regulation of HSV gene expression generally occurs at the transcriptional level (as opposed to translational) as demonstrated by the close correlation between the detection of mRNA transcripts and the kinetics of the expression of proteins encoded by them (Zhang & Wagner, 1987; Smith & Sandri-Goldin, 1988). Viral genes of all three kinetic classes are transcribed by the cellular RNA polymerase II (Costanzo *et al.*, 1977) and are expressed in a coordinate and sequential fashion during the course of productive infection (Hones & Roizman, 1975).

Immediate-early genes are the first to be expressed during the replicative cycle, being detected within 1 hr. pi (Hones & Roizman, 1975). The synthesis of early gene products is detectable shortly after the appearance of the IE proteins, but peaks later at around 4-6 hrs pa; early gene expression requires functional immediate-early proteins (Preston, 1979a; Dixon & Schaffer, 1980). Finally, the late genes are expressed, appearing at about 2 hrs after the onset of viral DNA replication; maximal expression of late genes requires the activities of both IE and E gene products as well as viral DNA synthesis (Holland *et al.*, 1980; O'Hare & Hayward, 1985). Late genes can actually be sub-divided into two further groups termed early-lates (or delayed-early) and true lates. The primary difference is that early-late gene expression does not require viral DNA synthesis (although viral DNA synthesis is required for maximal expression), whereas expression of

true-late genes is dependent upon viral DNA synthesis (Wagner, 1985, Roizman & Batterson, 1985).

In terms of general function, IE genes encode the major HSV regulatory proteins, early genes code for proteins involved in viral DNA synthesis, and the late genes specify virion structural proteins.

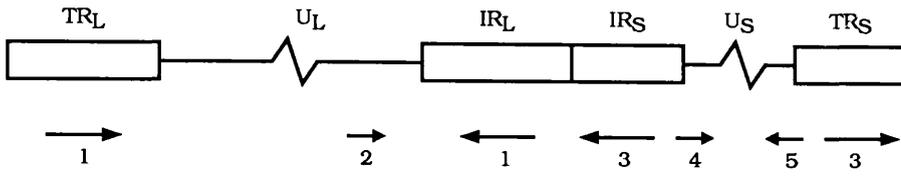
1B.2. The Immediate-Early Genes of HSV-1

Immediate-early genes are the only HSV-1 genes expressed in the absence of *de novo* protein synthesis, with their mRNAs accumulating to high levels under these conditions (Kozak & Roizman, 1974; Clements *et al.*, 1977; Jones & Roizman, 1979). ³²P-labelled IE-RNA transcripts were used to identify the positions of the IE genes on the virus genome, with *in vitro* translation being used to define their protein products (Watson *et al.*, 1979). HSV-1 encodes five IE mRNA species from five IE genes (IE175, IE110, US1, UL54 and US12); their genomic locations and orientations (Clements *et al.*, 1979), are shown in Figure 3.

Analysis of the IE genes by RNA mapping and DNA sequencing has revealed many interesting features concerning the structures of these genes (Clements *et al.*, 1979; Rixon *et al.*, 1982; Murchie & McGeoch, 1982; McGeoch *et al.*, 1985, 1986, 1988a; Perry *et al.*, 1986). Three of the five primary IE transcripts (IE genes 1,4 and 5) are spliced; a phenomenon that is particularly rare in HSV (Wagner, 1985). The IE-4 and IE-5 RNAs are derived from identical promoters located in the repeat sequences which bound U_s. The mRNA leader sequences and splice sites of these transcripts are located in the repeat sequences while their coding sequences are different, being located at either end of U_s (Watson *et al.*, 1979a; Rixon & Clements, 1982). The transcript of the IE-1 gene is also spliced and maps wholly within the R_L repeat sequences thereby making

Figure 3. A simplified map of the HSV-1 genome showing the positions and orientations of the IE genes. The internal and terminal repeat sequences are labelled IR and TR with the subscript denoting the unique segment with which they are associated. The IE genes are labelled 1 to 5. (Not to scale).

Table 2. Nomenclature and properties of the HSV-1 IE genes and their products. The Glasgow system of nomenclature for the HSV-1 IE genes and their products is shown with the sizes of the primary unmodified amino acid sequence deduced from the DNA sequence and the sizes of the polypeptides as determined from SDS-polyacrylamide gel electrophoresis.



| Gene | Product | Size on SDS PAGE | Size from Sequence |
|-------------|----------------|-------------------------|---------------------------|
| IE-1 | Vmw110 | 110kd | 78452 |
| IE-2 | Vmw63 | 63kd | 55376 |
| IE-3 | Vmw175 | 175kd | 132835 |
| IE-4 | Vmw68 | 68kd | 46521 |
| IE-5 | Vmw12 | 12kd | 9792 |

it diploid (Perry *et al.*, 1986). The IE-3 RNA is unspliced, and again diploid as it is transcribed entirely from within the R_s repeat sequences (Rixon *et al.*, 1982). The only IE gene that is not in any way associated with the repeat sequences is that of the IE-2 gene whose unspliced RNA maps in U_L (Whitton *et al.*, 1983).

It seems that there is a discrepancy between the rate of IE gene transcription and IE mRNA and protein accumulation (Weinheimer & McKnight, 1987). Nuclear run-on assays have demonstrated that while IE-3 gene transcription is turned off after 1 hr, the IE-1 and 4/5 genes are still transcribed with increasing rates up to 5 hrs post-infection. This observation is in contrast to the pattern of mRNA accumulation in the presence of metabolic inhibitors of mRNA and protein synthesis, which is similar for IE-1, 3 and 4/5 genes, thus suggesting that a post-transcriptional level of control must be regulating mRNA accumulation (Weinheimer & McKnight, 1987).

1B.3. Regulation of IE Gene Expression.

The control of transcription of the IE genes is an important area of research and a wealth of knowledge is accumulating concerning this subject. Expression from the HSV-1 IE promoters is regulated by the complex interactions of cellular factors and viral gene products which combine to interact at *cis*-acting elements upstream of the 5' ends of the IE-mRNAs. Indeed, the mechanism of IE gene *trans*-induction by virion/cellular factor interactions is among the best understood and has become a fine example of combinatorial control of eukaryotic transcription.

3.1. The IE-Promoters.

Comparison of the 5' flanking regions of IE genes

in HSV-1 and their counterparts in HSV-2 has revealed several discrete blocks of conserved DNA sequences (Whitton *et al.*, 1983; Whitton & Clements, 1984). The promoter regions of the IE genes, like many cellular genes, can be dissected into two components. The proximal component consists of TATA box homologies and IE-mRNA cap sites which are necessary for basal and induced levels of transcription. The distal component comprise multiple *cis*-acting elements which confer enhancer function and responsiveness to IE regulation (Mackem & Roizman, 1982a,b; Cordingley *et al.*, 1983; Preston *et al.*, 1984; Kristie & Roizman, 1984; Bzik & Preston, 1986). These distal elements include 5'-CCGCCC motifs that bind the purified cellular factor Sp1 *in vitro*, and these have been shown to be associated with basal expression properties of the IE-3 and 4/5 promoters (Jones & Tijan, 1985). Another conserved block is the consensus 5'-TAATGARAT motif (R=purine) which is present in several copies upstream from IE genes, usually beyond -110 bp from the mRNA cap site. Functional analysis has demonstrated that this element is essential for the responses to *trans*-induction by the virion associated polypeptide Vmw65 (Mackem & Roizman, 1982b; Preston *et al.*, 1984; Kristie & Roizman, 1985; Gaffney *et al.*, 1985; Bzik & Preston, 1986; O'Hare & Hayward, 1987). In contrast to the previously mentioned motifs, the TAATGARAT has not been found upstream from any of the HSV early or late genes^{αxcp1, UL39} (Wagner, 1985). DNA fragments which contain the TAATGARAT consensus can confer an IE pattern of regulation on the thymidine kinase gene (TK; an HSV early function) (Post *et al.*, 1981; Cordingley *et al.*, 1983) and plasmids with HSV IE upstream sequences from which the TAATGARAT motif has been deleted are unable to activate transcription from a linked promoter on superinfection (Preston *et al.*, 1984). A second IE promoter element which may contribute to the response to Vmw65, 5'-GCGGAAC, has also been described (Bzik & Preston, 1984;

Treizenberg *et al.*, 1988b) and possibly acts cooperatively with TAATGARAT.

The IE TAATGARAT motif overlaps with the 5'-ATGCAAAT octamer motifs which serve as binding sites for the ubiquitous cellular factor NF-III, also termed the octamer-binding transcription factor (OTF-1) (Pruijn *et al.*, 1986; Fletcher *et al.*, 1987) and indeed, NFIII has been demonstrated to bind TAATGARAT (O'Hare & Goding, 1988; Gerster & Roeder, 1988). This interaction is discussed more thoroughly in the next section.

Three of the five IE promoters have copies of the Vmw175 binding site ATCGTC which has been implicated in repression of the IE-3 promoter (Gelman & Silverstein, 1987, DeLuca & Schaffer, 1988). The IE-1 and 4/5 promoters harbour exact copies of the ATCGTC motif and bind Vmw175 (Faber & Wilcox, 1986b, 1988; Kristie & Roizman, 1986a,b; Muller, 1987). The IE-2 promoter contains a partial ATCGTC homology and is possibly bound by Vmw175 with weaker affinity (Kristie & Roizman, 1986a; Faber & Wilcox, 1988). However, Vmw175-mediated repression has only ever been convincingly shown for the IE-3 promoter (DeLuca & Schaffer, 1985; O'Hare & Hayward, 1985b, 1987; Gelman & Silverstein, 1986, 1987; Roberts *et al.*, 1988).

3.2. Trans-Activation of IE Genes by the Virion-Associated Trans-Inducing Factor, Vmw65.

Transcription from IE promoters is stimulated upon HSV infection by a virion tegument component identified as the major late phosphorylated protein Vmw65, known also as VP16 and α -TIF (Post *et al.*, 1981; Batterson & Roizman, 1983; Campbell *et al.*, 1984) and is present in the virion at approximately 400-600 molecules per particle (Heine *et al.*, 1974; Roizman *et al.*, 1975). The virion assembly and IE transactivation properties are separable and distinct

functions of Vmw65 (Ace *et al.*, 1988). This is demonstrated by the HSV mutant *in1814* containing a 12 bp insertion in the Vmw65 gene which assembles apparently normal virus particles, but is deficient for *trans*-induction (Ace *et al.*, 1989). The Vmw65 defect, like that in Vmw110 mutant *dl1403* (Stow & Stow, 1986; see also section 1B.4.4), is overcome by infection at high multiplicities. Under these conditions, expression of Vmw110 and Vmw63 is significantly reduced, Vmw68 is slightly affected, and Vmw175 expression is unaffected. At low moi of *in1814*, the expression of HSV-1 TK, an indicator early gene, is profoundly reduced, suggesting that IE gene expression is insufficient to activate the viral replication cycle. An HSV-2 mutant *ts2203* with a lesion near the 5' end of the corresponding gene causes a block in virion assembly at NPT, but does not affect IE transcriptional induction (Ace *et al.*, 1988).

The precise mechanism of *trans*-activation by Vmw65 has been a matter of intense investigation in recent years. Initial studies indicated that Vmw65 does not possess any intrinsic DNA binding properties, and that no other viral gene products were required for *trans*-induction of HSV IE gene expression. It therefore seemed likely that the TAATGARAT elements were the target for host-cell DNA-binding factors through which Vmw65 exerted its influence. (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare *et al.*, 1988). Indeed, it was subsequently demonstrated that the TAATGARAT element was recognised by a host cell factor termed variously α -H1 (Kristie & Roizman, 1987), H3 (Preston *et al.*, 1988) and TRF (O'Hare & Goding, 1988). The identification of a good octamer consensus motif overlapping many TAATGARAT elements, the identical gel mobility of a complex formed using octamer domain and TAATGARAT probes, and the ability of octamer probes to compete for the TAATGARAT-binding factor indicated that the host cell factor was the ubiquitous octamer-binding factor (O'Hare *et al.*, 1988). This transcription factor, known

variously as oct-1, OTF-1 and OBP100 (Singh *et al.*, 1986; Sive & Roeder, 1986; Bohmann *et al.*, 1987; Sturm *et al.*, 1987) is indistinguishable from the cellular factor NFIII which is involved in adenovirus replication (O'Niell *et al.*, 1988). It has now been verified that oct-1-NFIII is indeed the factor that binds the TAATGARAT elements (Baumrucker *et al.*, 1988; ApRhys *et al.*, 1989). The presence of Vmw65 in the infected cell complex formed at the TAATGARAT motif (the immediate-early complex; IEC) has been demonstrated by the recognition of IEC by a monoclonal antibody directed against Vmw65 (Preston *et al.*, 1988; O'Hare *et al.*, 1988). Also, the addition of purified or *in vitro* translated Vmw65 to uninfected cell extracts induces the formation of a complex identical to IEC (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare *et al.*, 1988; Gerster & Roeder, 1988). In the presence of Vmw65, the addition of purified oct-1 protein to oct-1 depleted extracts stimulates the formation of IEC (Gerster & Roeder, 1988), thereby demonstrating that both Vmw65 and oct-1 are part of the IEC.

The oct-1 and closely related lymphoid-specific oct-2 proteins are POU homoeodomain-containing proteins, characterized by an extensive region of homology called the POU domain which is responsible for their ability to bind DNA, and includes an N-terminal POU-specific region of 75 amino acids. Stern *et al.*, (1989) demonstrated that the oct-1 homoeodomain directs formation of the complex with Vmw65. Sequence comparisons (Laughon & Scott, 1984) and a structural analysis (Otting *et al.*, 1988) indicate that POU homoeodomains contain a tri- α -helical structure similar to the DNA binding domains of λ - and ϕ 434 repressors (Jordon *et al.*, 1988; Aggarwal *et al.*, 1988). Changing three of the amino acid residues on the surface of the homoeodomain that face away from the DNA disrupt complex assembly with Vmw65 without affecting DNA-binding activity (Stern *et al.*, 1989). Although affinity-purified oct-1 and oct-2 have been

demonstrated to bind the TAATGARAT element, only oct-1, is capable of forming a DNA-protein complex with Vmw65. Corresponding experiments with Vmw65 have demonstrated that deletions and single point mutations of residues in the region between 380 and 388 aa's abolish IEC formation; this region is now proposed to be intimately involved in protein-protein/ protein-DNA contacts within the IEC (Greaves & O'Hare, 1990).

Further investigation has revealed that more protein factors are involved in the IEC. In contrast to mutations in the TAAT bases of TAATGARAT (which abolish oct-1 DNA-binding), mutations in the GARAT motif still bind oct-1 but no longer support formation of a Vmw65/oct-1 complex (Gerster & Roeder, 1988; O'Hare *et al.*, 1988). Another intriguing observation is that highly purified oct-1 is inefficient at IEC formation (Gerster & Roeder, 1988) thus suggesting that other cellular factors are involved. Subsequent work with purified Vmw65 expressed from baculovirus has demonstrated that multiple cellular factors interact with Vmw65 to generate protein-DNA complexes more complex than Vmw65/oct-1 alone. At least an additional two as yet unidentified cellular factors are involved; one of the components, found in mammalian and insect cells and therefore probably a highly conserved and ubiquitous factor(s), is necessary for the formation of the Vmw65/oct-1 complex by highly purified Vmw65 and oct-1 (Kristie *et al.*, 1989). Mutations in GARAT preclude formation of this complex, thus implicating GARAT as a recognition site for this factor. Another component of the IEC has been identified in Hela cells, but not insect cells. Since the footprints of these complexes are similar, this additional factor probably represents a protein which binds the IEC purely through protein-protein interactions (Kristie *et al.*, 1989).

The *trans*-activation domain of Vmw65 is contained within the carboxy-terminal 79 amino acids. Deletions in

this region allow formation of the IEC, but fail to transactivate transcription (Triezenberg *et al.*, 1988a; Greaves & O'Hare, 1989). This domain can function independently of the remainder of the Vmw65 molecule as demonstrated by its ability to activate gene expression when fused to the DNA-binding domain of the yeast transactivator GAL4 (Sadowski *et al.*, 1988; Chasman *et al.*, 1989; Cousens *et al.*, 1989). Examination of the predicted secondary structure likely to be adopted by the carboxy-terminal amino acids indicates that they probably form a highly negatively charged amphipathic α -helix (Cousens *et al.*, 1989). Similar structures have been identified in the transcription activation domains of the yeast proteins GAL4 (Gill & Ptashne, 1987) and GCN4 (Hope & Struhl, 1986), and are likely to be present in many although not all eukaryotic transcription factors. This acidic amphipathic α -helix probably provides a surface for intimate protein-protein interactions with other components of the transcription machinery such as TFIID, the TATA box binding factor or RNA polymerase subunits. Indeed, when used as a column ligand for affinity chromatography, the transactivation domain of Vmw65 binds strongly and highly selectively to the human and yeast TATA box-binding factors thus implying that the principal target of acidic activation domains in general is the TATA-box factor TFIID (Stringer *et al.*, 1990; see section 1C.7).

1B.4. HSV IE GENE PRODUCTS

Five IE gene products have been identified in HSV-infected cells (see section 1B.2), and information regarding the IE proteins is displayed in Table 2. The IE proteins, with the exception of Vmw12, are all phosphorylated, found predominantly in the nucleus of infected cells, and bind DNA in crude extracts (Pereira *et al.*, 1977; Marsden *et al.*, 1978; Hay & Hay, 1980; Ackermann

et al., 1984). Their physical properties and kinetics of expression make them strong candidates as regulators of the virus life cycle, and indeed, four of the five HSV IE gene products are implicated in viral gene regulation. However, only two are absolutely essential for viral multiplication (reviewed by Everett, 1987a). The investigation of the role of IE gene products during viral replication, particularly Vmw175, has been greatly facilitated by the isolation of mutant viruses with temperature-sensitive (*ts*) lesions or deletions in the IE polypeptides. The following sections review the properties of each IE gene product.

4.1. Vmw12

Vmw12 is the least understood IE protein. Unlike the other IE gene products, it is non-phosphorylated and cytoplasmically localized (Preston, 1979b; Marsden *et al.*, 1982). The isolation of viable deletion mutants which lack the whole of this gene demonstrates that it does not play an essential role during infection of tissue culture cells (Longnecker & Roizman, 1986; Umene, 1986; Brown & Harland, 1987).

4.2. Vmw68

A deletion mutant which produces only the carboxy-terminal third of Vmw68 (the product of IE-gene 4) has been constructed (Post & Roizman, 1981). Although this virus is viable in tissue culture, it is impaired for late gene expression and grows poorly in some cell types (Sears, 1985). The mutant virus was not neurovirulent, but could establish latency in mice. Vmw68 may therefore play a role in late gene regulation and possibly replaces a cell type restricted host factor in some cell types. These studies have as yet failed to provide an answer to the role of Vmw68, and a more complete study is required to determine its true function.

4.3. Vmw63

The product of IE-gene 2, Vmw63 is an essential regulatory protein. HSV-1 mutants with *ts* lesions in the Vmw63 gene exhibit reduced expression of many early-late genes and are unable to induce expression of many true-late genes at NPT (Sacks *et al.*, 1985). Studies of other mutants have indicated that Vmw63 is required to repress expression of certain IE and early genes and is absolutely required for the transcriptional activation of late genes; a function that is independent of early protein and viral DNA synthesis (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice *et al.*, 1989).

In contrast to the activities of Vmw110 and Vmw175 (see below), Vmw63 alone exhibits little regulatory activity for HSV promoters in transient expression assays (DeLuca *et al.*, 1985; Everett, 1984b; Sekulovich *et al.*, 1988). An exception though is that Vmw63 by itself can *trans*-activate the early-late glycoprotein B (gB) promoter (Rice & Knipe, 1988). It has also been shown to be able to up-regulate expression of a retroviral vector containing Moloney murine leukemia virus regulatory sequences (Chapman *et al.*, 1992). Despite its limited regulatory activity alone, Vmw63 can both repress and enhance expression from a variety of HSV-1 promoters whose expression is induced by Vmw110 or Vmw175 or both (Everett, 1986; Rice & Knipe, 1988; Sekulovich *et al.*, 1988; Su & Knipe, 1989). This indicates that Vmw63 may act as a modulator of the regulatory functions of Vmw110 and Vmw175, although how this is achieved is, as yet, unknown.

Mutational analysis and transient expression assays have been employed to assign the functional activities of repression and enhancement to the Vmw63 molecule. These studies have indicated that two functional domains responsible for Vmw63-mediated repression lie within the hydrophobic carboxy-terminal one-third of the molecule, and that the carboxy-terminal half of Vmw63 is required for

enhancing activity (McMahon & Schaffer, 1990). Studies utilising mutant viruses with in-frame deletion and *ts* lesions in Vmw63 have indicated that Vmw63 is responsible for a distinct shift in mobility of Vmw175 in SDS gels, implicating Vmw63 in post-translational modification of Vmw175 (Sacks *et al.*, 1985; Rice & Knipe, 1989; McMahon & Schaffer, 1990). Vmw63 also performs its repressing and enhancing functions at different times during infection; it functions as an enhancer of viral gene expression at early times of infection and as a repressor of early gene expression and enhancer of late gene expression at later stages of the HSV-replication cycle (McMahon & Schaffer, 1990).

4.4. Vmw110

As yet, no *ts* mutants in IE-gene 1 have been isolated, and evidence suggests that this gene is not absolutely essential for virus growth in tissue culture. Reduction of IE-1 mRNA to less than 10% of normal levels has no effect on virus gene expression or virus yield (Sandri-Goldin *et al.*, 1987). Also, virus mutants with large deletions within both copies of the IE-1 coding sequence are viable in tissue culture. They grow very poorly at low multiplicities of infection (moi), although this defect is overcome at high moi. (Stow & Stow, 1986; Sacks & Schaffer, 1987).

Transfection experiments have demonstrated Vmw110 to be a promiscuous transactivator of both HSV and heterologous promoters, acting independently and in synergy with Vmw175 (Everett, 1984b, 1986; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985a, b; Quinlan & Knipe, 1985). Although Vmw110 can be shown to have a substantial effect in such assays, its role during normal virus infection is an unresolved question. It has been demonstrated to confer strong growth advantage to the virus, however, despite being a potent activator of gene expression, it cannot

complement the activities of Vmw175 in viruses carrying defects in the IE-3 gene (Deluca & Schaffer, 1985, 1988; Russel *et al.*, 1987b).

The predicted Vmw110 polypeptide contains 775 amino acids, and two introns are spliced out of the coding region of the primary transcript to form the mature mRNA (Perry *et al.*, 1986). One particular region of the protein has been predicted to have an interesting predicted secondary structure motif; a region from residues 99-156 containing 9 cysteine residues, including 3 C-n-n-C pairs which are proposed to form a zinc-finger binding domain (Berg, 1986). Such regions are predominantly involved in nucleic acid binding, although their involvement in protein-protein interactions has also been noted (Frankel & Pabo, 1988). Vmw110 also contains a serine-rich domain from residues 554-594 which could potentially form a site of phosphorylation of the protein.

Mutagenesis and transient transfection studies have revealed that the Vmw110 polypeptide can be divided into at least 5 regions important for transcriptional activation of the gD promoter in synergy with Vmw175 (Everett, 1987b, 1988). The regions important for Vmw110 activation in the absence of Vmw175 however are somewhat different, implying that these two activities are distinct functions of the Vmw110 polypeptide. The predicted zinc finger domain is important for Vmw110 activity alone, but not in the presence of Vmw175, whereas the carboxy-terminal region is important for synergistic, but not independent activity.

The nuclear localization signal of Vmw110 has been shown to lie between residues 475-638 and contains a highly basic sequence similar to other similarly identified signals (Kalderon *et al.*, 1984; Krippel *et al.*, 1985; Everett, 1988).

The growth properties of recombinant HSV-1 viruses encoding insertion and deletion mutants have been examined (Everett, 1989). Deletions in the cysteine-rich region

severely impaired virus growth, while lesions in the C-terminal region proved to be less important, thus indicating that the putative zinc-finger is important for the biological role of Vmw110 in the HSV-1 lytic cycle.

Despite the detailed functional analysis of Vmw110, the exact mechanism of Vmw110 action and its precise function remain poorly understood although, as with Vmw63, it clearly has a function in committing virus infection to the lytic cycle. In this respect, the role of Vmw110 in the establishment and reactivation of latency is now coming under intense scrutiny.

4.5. Vmw175

The product of IE-gene 3 has an apparent molecular weight of 175 kd although the size of the unmodified protein is 132,835 (McGeoch *et al.*, 1986). Mutational analysis of Vmw175 has demonstrated the central role of this polypeptide in the virus growth cycle. Viruses with *ts* lesions in Vmw175 are generally inefficient or completely unable to activate transcription of later classes of genes, while overexpressing IE functions (Preston, 1979a; Dixon & Schaffer, 1980; DeLuca *et al.*, 1984; Preston, 1981). From this, it was concluded that functional Vmw175 is required for transcription of later classes of viral genes and is also directly or indirectly involved in the autoregulation of IE gene transcription (Preston, 1979b; Dixon & Schaffer, 1980; Watson & Clements, 1980). The mapping of these mutations has revealed that many lie towards the carboxy-terminal of the protein while others (including *tsK*) are in the central region of the gene (Dixon & Schaffer, 1980; Preston, 1981). The IE-gene 3 *tsK* lesion has been identified as a C-T transition causing an alanine to valine change in a region which exhibits a high level of homology with the corresponding VZV 140K protein (Davison *et al.*, 1984; McGeoch *et al.*, 1986). Other *ts* lesions of IE-3 which overproduce Vmw175 and Vmw63 and synthesise many early but

no late polypeptides map to the carboxy-terminal end of the protein (DeLuca *et al.*, 1984).

Vmw175 is post-translationally modified both by phosphorylation and poly(ADP)ribosylation and migrates as multiple species on SDS polyacrylamide gels (Pereira *et al.*, 1977; Preston & Notarianni, 1983). Partially purified Vmw175 has been obtained and appears to exist in the form of a homodimeric complex (Metzler & Wilcox, 1985). The predicted primary sequence of Vmw175 can be divided into five regions on the basis of similarity to the corresponding proteins expressed by other alphaherpesviruses (McGeoch *et al.*, 1986; Grundy *et al.*, 1989; Vlcek *et al.*, 1989). Regions 2 and 4, composed of residues 315-484 and 797-1224 respectively, are the most highly conserved and include the locations of several *ts* lesions (Davison *et al.*, 1984; DeLuca *et al.*, 1984; Paterson *et al.*, 1990). In addition, the construction of insertion mutants in a plasmid-encoded copy of IE-3 has demonstrated that mutations in regions 2 and 4 affect the ability of Vmw175 to regulate gene expression in transfection assays (Paterson & Everett, 1988).

A major step in understanding the mechanism of Vmw175 action has been the demonstration that it can bind to DNA sequences containing a consensus ATCGTC motif (Beard *et al.*, 1986; Faber & Wilcox, 1986b; Muller, 1987; Kattar-Cooley & Wilcox, 1989; Tedder *et al.*, 1989) and also, although probably less strongly, to other less well characterized sequences (Michael *et al.*, 1988). The ATCGTC motifs, however, are not located in promoter regions of early or late promoters, although an exception is an ATCGTC motif found 100 bp upstream of the 5' end of the gD mRNA which has been implicated in optimal stimulation of the gD promoter by Vmw175 *in vitro* (Tedder *et al.*, 1989). How this Vmw175 DNA-binding activity is related to the transcriptional activation mechanism of early and late promoters is unknown. Strong Vmw175 DNA-binding activity at

the IE-gene 3 cap site has however been implicated in the mechanism of autoregulation, both in transfection assays (Roberts *et al.*, 1988) and in the viral genome (DeLuca and Schaffer, 1988). It has been postulated that such binding to the IE-3 promoter may impede either the passage of RNA polymerase or the formation of transcriptional pre-initiation complexes, thus preventing expression of the IE-3 gene.

Mutational analysis of Vmw175 has demonstrated that the principal part of the polypeptide required for DNA-binding lies within region 2 (DeLuca & Schaffer, 1988; Paterson & Everett, 1988; Shepard *et al.*, 1989). The Vmw175 polypeptide encoded by HSV-1 *ts1225* is able to bind DNA *in vitro* at temperatures at which it fails to autoregulate *in vivo* thus demonstrating that DNA-binding and autoregulation are distinguishable activities of the Vmw175 polypeptide, and DNA-binding *per se* is insufficient for autoregulation *in vivo* (Paterson *et al.*, 1990). It has also been noted that HSV-1 IE-3 mutants unable to bind DNA are partially permissive for early gene transcription (DeLuca *et al.*, 1984; Paterson *et al.*, 1990), which suggests that the ability of Vmw175 to bind to at least one class of recognition sequences *in vitro* does not correlate with the activation of early genes during infection.

Like the other IE-genes of HSV, the actual mechanism of transcriptional regulation by Vmw175 remains an enigma. Further insight into this phenomenon awaits further analysis of Vmw175 DNA-binding, and a study of the interactions between Vmw175 and the host transcriptional machinery. In this later respect, TFIID remains the obvious target for such an interaction (cf. the PRV IE polypeptide (p.57)).

1B.5. Regulation of Early Gene Expression

The study of early gene expression has centred predominantly upon the analysis of promoter DNA sequence requirements for the activation of transcription in *trans*

by viral gene products. The best studied examples in this respect are the promoters of the tk gene (UL23) and gD gene (US6) which have both been dissected using a variety of techniques. These experiments generally involved mutagenesis of the E promoter region and quantitation of their activity by their ability to drive expression of a linked reporter gene when injected into *Xenopus* oocytes (McKnight *et al.*, 1981, 1982; Jones *et al.*, 1985) or transfected into eukaryotic cells (Everett, 1983, 1984b). Additionally, mutagenized E (tk gene) promoter sequences have been introduced back into the HSV-1 genome and assayed for their ability to drive gene expression during virus infection (Coen *et al.*, 1986).

These approaches demonstrated that the tk promoter consists of 110 bp (McKnight & Kingsbury, 1982) and the gD promoter of 83 bp (Everett, 1983) immediately upstream from the RNA capsite. The functional regions of these promoters consist of a consensus CAAT box and GC- (Sp1 binding) or GA-rich motifs in their distal promoter regions and a proximal TATA box. These sequence elements have been shown to be required for their *trans*-activation by both Vmw175 and Vmw110 (Everett, 1984a; Eisenberg *et al.*, 1985; Coen *et al.*, 1986; Jones *et al.*, 1985). In addition, mutations in the capsite region result in a moderate reduction in promoter activity (Everett, 1984b). Studies of the tk and gD promoters did not identify sequences required for viral *trans*-activation which were not needed for *cis*-activation (Everett, 1984b; Eisenberg *et al.*, 1985; Coen *et al.*, 1986) suggesting that IE products activate E gene transcription either by binding sequences recognized by cellular transcription factors or by altering the activity of cellular transcription factors.

Analysis of linker scanning mutations of the tk gene locus in the HSV-1 genome (Coen *et al.*, 1986) demonstrated that the same promoter elements were important for activity in the virus genome as in the short term

transfection assays. However, the upstream distal promoter elements were shown to be quantitatively less crucial than the TATA box and cap site. Interestingly, mutations downstream of the RNA capsites have also shown to cause a reduction in tk promoter activity during virus infection (Coen *et al.*, 1986; Zipser *et al.*, 1981). However, since deletion mutants in this region still express appreciable amounts of tk RNA (Halpern & Smiley, 1984), it was suggested that this region affected the binding of cellular factors to the TATA box rather than being a previously unidentified promoter domain *per se* (Coen *et al.*, 1986).

1B.6. Regulation of Late Gene Expression

The late genes of HSV-1 require DNA replication for their maximal expression with L gene products reaching a maximum by 10h-16h post adsorption (Hones & Roizman, 1973). The requirement of Vmw175 and Vmw110 for activation of L gene transcription is not yet clear although activation of the UL44 (gC) promoter by cotransfection with IE genes has been detected in a transient assay system (DeLuca & Schaffer, 1985; Mavromara-Nazos *et al.*, 1986).

The regulation of the true late gene US11 promoter (Johnson *et al.*, 1986) has been studied in some detail and has been shown to require DNA replication for its maximal expression, even when present on a plasmid (Johnson & Everett, 1986a). The promoter sequence elements of the US11 promoter were delineated using a plasmid containing an HSV origin of replication (*oris*) and the US11 promoter linked to the rabbit beta globin gene (Johnson & Everett, 1986a). This plasmid was transfected into Hela cells and then all the viral gene products required for DNA replication were provided by infection of the transfected cells. Surprisingly, it was discovered that the only sequences required for fully efficient regulated expression of the US11 gene consisted of a 31 bp region containing the proximal TATA box and RNA capsite. A study of the promoter

region of the gC gene gave similar results (Homa *et al.*, 1986b). These findings were confirmed by the demonstration that an early HSV-1 gene (gD) could be converted to a late gene by the removal of sequences 5' of its TATA box (Johnson & Everett, 1986b).

1C. EUKARYOTIC RNA POLYMERASE II TRANSCRIPTION

1C.1. RNA Polymerase II Promoters

In eukaryotes, the promoters of protein coding genes are composed of several distinct elements usually located upstream of the start point of transcription. These include proximal promoter elements, distal promoter elements and enhancers (reviewed by Breathnach & Chambon, 1981; Nakajima *et al.*, 1988). The most common promoter element is the TATA box motif located around -30 from the mRNA cap site. Results *in vitro* and *in vivo* indicate that this is a critical motif for both promoter activity and determining the exact point of initiation. Moreover, for some promoters, the TATA element (sometimes with sequences near the initiation site) is the only DNA sequence required for a low level of accurate transcription *in vitro* (Corden *et al.*, 1980; Grosveld *et al.*, 1981) and for mediating transcription responses to HSV immediate-early proteins both *in vivo* (Green *et al.*, 1983; Coen *et al.*, 1986), and *in vitro* (Abmayr *et al.*, 1985, 1988). The TATA box represents a core element and cellular factors exist which functionally interact with this. The activities of core promoters can be greatly enhanced by the action of distal elements; upstream promoter elements act at a short distance while enhancer elements can be located up to several thousand base pairs from the transcription initiation site (Dyran & Tijan, 1985).

The various components of the human RNA polymerase II transcription machinery are being elucidated by the biochemical dissection of crude cell-free systems (Matsui *et al.*, 1980; Samuels *et al.*, 1982; Dignam *et al.*, 1983; Sawadogo & Roeder, 1985b). This approach has revealed that specific transcription initiation by RNA polymerase II requires the coordinated interaction of multiple protein factors. These can be divided into two categories: general transcription factors and upstream element binding proteins

(Dyran & Tijan, 1985). Our most detailed understanding has centred around the factors which interact directly with the core promoter elements. Crude extracts of mammalian cells have been broken down into at least five fractions (originally TFIIA through TFIIE) that are required by RNA polymerase II for the accurate transcription of a DNA template. As TFIIC has been found to be an artifact and TFIIE has been split into TFIIE and TFIIF, we are left with TFIIA, IIB, IID, IIE and IIF. An enormous amount of effort is now being put into identifying the function of these factors, the cloning of their genes and how they interact with each other and RNA polymerase II.

Due to the vast body of literature accumulating concerning RNA polymerase II transcriptional control, it is outwith the scope of this thesis to enter into all aspects of this rapidly advancing field in great detail. However, the following sections will deal with the more interesting aspects of RNA polymerase II, the general transcription factors, how they interact with each other, and how upstream factors interact with the core promoter complex.

1C.2. RNA Polymerase II

Eukaryotic cells contain three distinct forms of nuclear DNA-dependent RNA polymerases that transcribe different sets of genes. Transcription studies with reconstituted RNA polymerase dependent systems has provided firm *in vitro* evidence that RNA polymerase I synthesises ribosomal RNA precursors, while RNA polymerase II transcribes protein-coding genes and RNA polymerase III transcribes 5S and tRNA genes. In view of its pivotal role in gene expression, most studies have concentrated on RNA polymerase II.

RNA polymerase II has now been purified to near homogeneity from more than 20 different organisms; from animal and plant tissues, cultured cells and lower eukaryotes (Sawadogo & Sentenac, 1990). Such studies have

demonstrated that RNA polymerase II is a multi-subunit enzyme of molecular mass 500,000-600,000 consisting of two large polypeptides ($M_r > 140,000$) and a number of smaller polypeptides ranging in size from 10kd to 40kd (Sawadogo & Sentenac, 1990). Three different forms of RNA polymerase have been described that differ only in the apparent M_r of their largest subunit. These enzymes, termed IIO, IIA and IIB which contain large subunits of M_r 240,000, (IIo), 190-220,000 (IIa) and 170-180,000 (IIB) respectively have been shown to be the products of a single gene in yeast (Ingles *et al.*, 1984), *drosophila* (Ingles *et al.*, 1983) and mouse and human (Cho *et al.*, 1985).

An important discovery in the analysis of the class II transcription system was the discovery of an unusual carboxy-terminal extension of the IIA and IIO subunits in the yeast and mouse enzymes (Corden *et al.*, 1985). The sequence of this gene revealed that the C-terminal domain (CTD or tailpiece) consists of tandem repeats of the consensus heptapeptide sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) occurring 26 times in the yeast and 52 times in the mammalian enzyme. The importance of this novel domain has been demonstrated by genetic experiments whereby CTD deletions in the yeast and *drosophila* enzymes were shown to be lethal (Allison *et al.*, 1988; Zehring *et al.*, 1988) and a mouse enzyme with such a deletion was non-functional in rodent cells (Bartolomei *et al.*, 1988). A series of indirect experiments have implicated a role for the CTD in the initiation of RNA polymerase II transcription. Early experiments demonstrated that a monoclonal antibody recognising the IIA enzyme, but not the IIB form (which lacks the CTD) prevented accurate initiation of transcription of a variety of promoters *in vitro* (Dahmus & Kedinger, 1983). Another such monoclonal antibody also inhibited the formation of stable initiation-competent complexes at the adenovirus major late promoter (MLP) *in vitro* (Allison & Ingles, 1989). A role for the repeats in

initiation is, however, ambiguous since the *drosophila* RNA pol II with a deleted CTD can still initiate accurate transcription *in vitro* (Zehring *et al.*, 1988)

It has been proposed that the acid activation domains of eukaryotic DNA-binding transcription factors may regulate RNA pol II through direct or indirect interactions with the CTD (Allison *et al.*, 1988; Nonet *et al.*, 1987). Indeed, it has been demonstrated that mutant RNA pol II with a shorter or extended tailpiece can accentuate or suppress respectively, the deficiency of mutant GAL4 proteins or GAL4-GCN4 chimeric proteins to activate transcription *in vivo* (Allison & Ingles, 1989). However, there remain some conflicting observations. For example, substitution mutations and construction of chimeric tailpieces have indicated that all repeats are not functionally equivalent, suggesting some sequence specificity (Allison *et al.*, 1988). Moreover, while GAL4 is functional in *drosophila* and mammalian cells (Fischer *et al.*, 1988; Kakidani & Ptashne, 1988), the hamster CTD can replace the yeast CTD *in vivo*, whereas the *drosophila* domain will not (Allison *et al.*, 1988). Interestingly, Brandl & Struhl (1989) demonstrated that GCN4 can interact directly and specifically with yeast RNA pol II, but the basic carboxy-terminal DNA-binding domain of GCN4 (not the acidic activating domain) is sufficient for this interaction.

While the actual role of the CTD remains highly conjectural, it seems that it may be engaged in sequence-specific types of interaction with other enzyme subunits or a general transcription factor.

1C.3. The General Transcription Factors

The development of soluble extracts capable of reproducing *in vitro* the accurate transcription of class II promoters has been a major breakthrough for investigating both the general and regulatory mechanisms of transcription

initiation by RNA pol II. Crude extracts have currently been broken into at least five fractions, TFIIA, IIB, IID, IIE and IIF. The following subsections summarize the current knowledge concerning each factor.

TFIID. TFIID is the pivotal factor in the basal transcription apparatus, and independently binds to an approximately 10 bp sequence centered around the TATA motif. *In vitro*, the binding of TFIID to the TATA box is a prerequisite for the binding of other factors and RNA pol II to form an active transcription complex (Buratowski *et al.*, 1989; Davison *et al.*, 1983; Sawadogo & Roeder, 1985b; Van Dyke *et al.*, 1988) and for the *in vitro* transcription of several different genes (Nakajima *et al.*, 1988). Workman & Roeder, (1987), demonstrated that the binding of TFIID can be inhibited by the presence of nucleosomes over the TATA region, suggesting that the state of chromatin in the promoter region can influence transcriptional initiation by its effect on TFIID binding.

TFIID is functionally conserved among eukaryotes (Buratowski *et al.*, 1988; Cavallini *et al.*, 1988) as demonstrated by the interchangeability of the yeast and mammalian TFIID in an *in vitro* transcription system. However, as the mammalian factor is highly labile and difficult to purify (Nakajima *et al.*, 1988) most investigation has centred around the yeast protein. The gene encoding TFIID has now been cloned from yeast (Schmidt *et al.*, 1989; Hahn *et al.*, 1989; Eisenmann *et al.*, 1989; Horikoshi *et al.*, 1989; Cavallini *et al.*, 1989), *drosophila* (Hoey *et al.*, 1990) and humans (Cheng Kao *et al.*, 1990; Peterson *et al.*, 1990). These genes encode proteins of 240 aa (27 kd), 353 aa (38 kd) and 339 aa (37 kd) respectively. The TFIID gene products of yeast and *drosophila* were demonstrated to be the products of single unspliced genes, a finding which dispelled speculation that different TFIID's may have existed for interaction with functionally

distinct TATA elements.

Sequence analysis of these genes has failed to demonstrate any degree of homology to any other previously identified protein. Comparison of their sequences has revealed that the TFIID protein consists of a bi-partite structure, comprising a highly conserved 181 amino acid carboxy-terminal domain and a non-conserved amino-terminal domain, varying in size in a species-dependent manner. A truncated TFIID protein consisting of only 191 of the carboxy-terminal amino acids was able to bind DNA as effectively as the full length protein, and could activate basal-level transcription (Hoey *et al.*, 1990), thus demonstrating that the carboxy-terminal domain is a 'core' conserved sequence required for basal level activity. While the carboxy-terminal region is rich in basic amino acids, no sequence similarity with any other previously identified DNA-binding motifs has been detected (Johnson & McKnight, 1989). Interestingly however, there are two copies of a 34 aa motif present in both yeast and *drosophila* (Cavallini *et al.*, 1989; Hoejimakers, 1990; Nagai, 1990) which occur in a region which shares weak homology with prokaryotic sigma factors (Hahn *et al.*, 1989; Horikoshi *et al.*, 1989). Sigma, a loosely associated component of the bacterial RNA polymerase, is required for accurate initiation of transcription, and contains diverse activities such as RNA polymerase binding, promoter recognition and DNA melting (Helmann & Chamberlin, 1988).

The lack of similarity between the amino-terminal domains suggests that they may have evolved to satisfy functions specific to each species. The role of this domain and its interaction with the regulatory transcription apparatus is discussed in section 1C.7.

TFIIA. The extent, or the involvement of TFIIA in RNA pol II initiation is highly controversial. The requirement for this factor is dependent on its source and degree of

purification. The *in vitro* requirement for TFIIA on the synthesis of specific transcripts ranges from no requirement (Sawadogo & Roeder, 1985b; Van Dyke *et al.*, 1988) to a stimulatory effect (Egly *et al.*, 1984; Buratowski *et al.*, 1989) or even an absolute requirement (Samuels & Sharp, 1986; Reinberg *et al.*, 1987). Kinetic experiments seem to indicate a function for TFIIA before (Reinberg *et al.*, 1987) or simultaneously with (Egly *et al.*, 1984; Samuels & Sharp, 1986) the interaction of TFIID with the TATA box, and probably acts to enhance the TFIID/TATA box interaction. Interestingly, there is no TFIIA requirement for the footprinting of yeast and human TFIID (Nakajima *et al.*, 1988) while it is absolutely required for gel shift analysis (Buratowski *et al.*, 1989). TFIIA has been purified either as a 43 kd protein (Egly *et al.*, 1984) or as a series of polypeptides ranging in molecular weight from 12,000 to 19,000 from calf thymus (Samuels & Sharp, 1986).

TFIIB. The TFIIB protein is essential for transcription of a number of RNA pol II genes (Conaway *et al.*, 1987; Zheng *et al.*, 1987). TFIIB has been purified to near homogeneity from human and rat cells as single polypeptides of 35 kd and 27 kd respectively (Conaway *et al.*, 1987; Zheng *et al.*, 1987). Interestingly, TFIIB and TFIIE have been shown to interact directly with each other as determined by their co-sedimentation on a glycerol gradient (Zheng *et al.*, 1987). Neither of these factors recognizes promoters directly, nor do they facilitate preinitiation complex formation (Conaway & Conaway, 1989). Rather, it seems that the interaction of TFIIB and E with transcription initiation complexes may be driven by their intrinsic affinity for RNA polymerase and/or other transcription factors.

TFIIE/F. TFIIE is a heterotetramer containing two subunits of relative molecular mass 57,000 and two of 31,000 (Conaway & Conaway, 1989; Peterson *et al.*, 1991). The genes encoding both subunits have now been cloned (Ohkuma *et al.*, 1991; Peterson *et al.*, 1991; Sumimoto *et al.*, 1991) and appear to be related to bacterial sigma factors. They also contain several interesting structural motifs including helix-loop-helix, leucine repeat and zinc finger motifs. Flores *et al.*, (1989) also reported the separation of TFIIE into two fractions termed TFIIE and TFIIF which are both required in their reconstituted system for specific transcriptional initiation of a number of cellular genes. In addition, they demonstrated that these two activities could individually interact with RNA polymerase during glycerol gradient centrifugation (Flores *et al.*, 1988). TFIIF is thought to be related to the RAP30/74 complex demonstrated by Sopta *et al.*, (1985) to have very strong affinity for RNA polymerase. RAP30/74 is required for the formation of the first phosphodiester bond in nascent RNA (Burton *et al.*, 1988) and is absolutely required for initiation. RAP30/74 also has an ATP dependent DNA helicase activity (Sopta *et al.*, 1989).

RAP30 has now been cloned and shown to be a 234 aa protein of apparent molecular weight 26,312 (Sopta *et al.*, 1989). It was demonstrated that RAP30 was not the helicase, leaving RAP74 as the most likely candidate for this function. It has also been shown that RAP30 binds to RNA polymerase II and prevents it from binding nonspecifically to DNA (Killeen & Greenblatt, 1992). Sequence analysis of RAP30 has indicated homology with bacterial sigma factor. It seems likely that TFIID and RAP30 each represent one portion of sigma, thus implying that the diverse activities of sigma may be distributed among several eukaryotic factors. It would therefore seem reasonable to suppose that TFIID bears homology to the sigma region thought to contact the TATA-like sequences of prokaryotic promoters (Horikoshi

et al., 1989) and that RAP30 is similar to the sigma region which binds bacterial RNA polymerase (Sopta *et al.*, 1989).

1C.4. Mechanism of Transcription Initiation by RNA Pol II

Various approaches have been used to identify and characterize intermediate steps in the complex reaction pathway leading to specific transcription initiation by RNA pol II. The most informative studies so far are those using direct physical methods such as footprinting analysis (Van Dyke *et al.*, 1988) and native gel electrophoresis (Buratowski *et al.*, 1989) which have allowed the characterization of various RNA pol II transcription complexes and their polypeptide compositions. Buratowski *et al.*, (1989) resolved a series of complexes by native gel electrophoresis that suggested an ordered assembly of the general transcription factors and RNA pol II on an Adenovirus-2 MLP template. The proposed complexes representing intermediates in the initiation reaction are represented in Figure 4 and are described in the following subsections.

Complex 1. The first step in initiation is the recognition of the TATA box by TFIID. Footprinting analysis demonstrated a protected region spanning -37 to -17 with yeast TFIID and -38 to -4 with Hela TFIID. This complex is undetectable in gel shift assays, and may therefore be unstable.

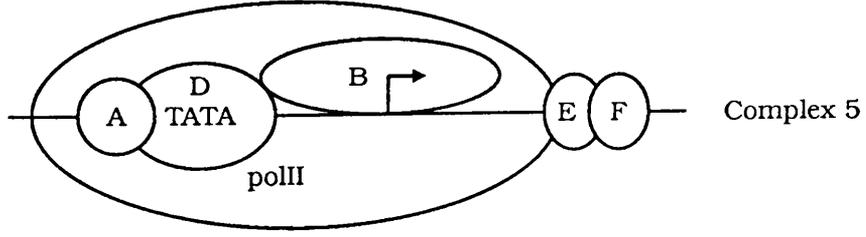
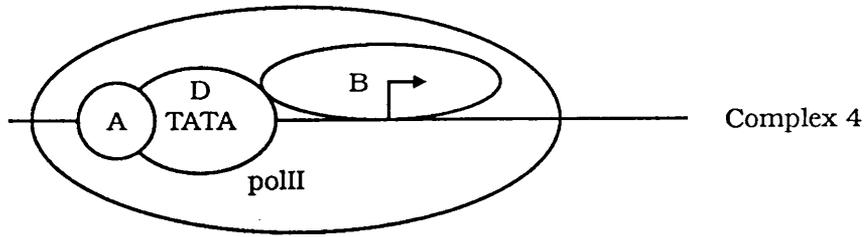
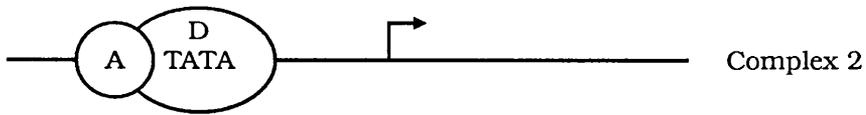
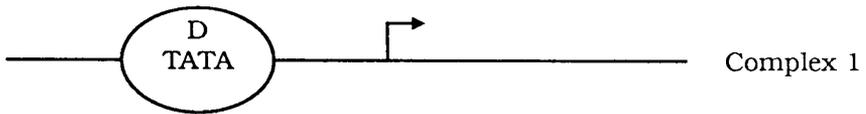
Complex 2. The addition of TFIIA enhances the binding of TFIID to the TATA element and modifies the upstream region of the footprint (-42 to -17). This complex is detectable in gel shift assays. It is possible that TFIIA may bind TFIID before interaction with the TATA box. (Reinberg *et al.*, 1987).

Complex 3. TFIIB is probably the next factor in the

Figure 4. A model for preinitiation complex assembly. RNA polymerase II (polII) and the general transcription factors TFIID (D), TFIIA (A), TFIIB (B), TFIIE (E), and TFIIIF (F), are assembled into a preinitiation complex on the DNA template. Buratowski *et al.* (1989) identified a minimum of five intermediate complexes leading to a functional preinitiation complex *in vitro*. The order in which the components are recruited onto the template and their relative positions within each complex are shown as determined; this is discussed more thoroughly in section 1C.4. The transcription start site (+1) is marked by the arrow, and the numbering at the top of this Figure refers to nucleotide positions relative to this.

This Figure is adapted from an original Figure presented in Buratowski *et al.* (1989).

-50 -40 -30 -20 -10 +10 +20 +30



initiation complex and is absolutely required for the formation of all other complexes. Addition of TFIIB to TFIID confers additional protection of the coding strand from -10 to +10, suggesting that it may be associated with one strand and extends beyond the transcription initiation site. TFIIB is also required for the subsequent binding of RNA polymerase; it has also been noted that TFIIB can be associated with RNA polymerase in solution (Zheng *et al.*, 1987). It therefore seems likely that TFIIB acts as a 'binding' molecule between the TFIIA-TFIID-TATA complex and pol II. It has also been proposed to be involved in measuring the distance from the complex to the initiation site. In this respect it would be interesting to observe what happens when mammalian TFIIB is replaced by its yeast homologue, since in *S. cerevisiae*, the initiation site is generally at a greater distance (60-120 bp) from the TATA motif than in higher eukaryotes (Guarente, 1987).

Complex 4. This complex is generated by the addition of RNA pol II to complex 3, and exhibits extensive DNase protection (-47 to +20). If the proximal protection is due to the polymerase, then this would allow direct interaction with USF (the MLP transcription factor) with binding site -50 to -66 (Van Dyke *et al.*, 1988). All pol II complexes occur in the form of pairs of bands on gel shift assays, but have identical footprints. This may be due to the binding of different forms of pol II, the binding of another protein to the complex (a candidate being the transcription elongation factor S-11 (Sopta *et al.*, 1985), or it may be that the mobility change may reflect a change in pol-DNA interaction such as observed with *E. coli* RNA polymerase (Straney & Crothers, 1985).

Complex 5. Generated by the addition of TFIIIE/F; these factors bind downstream of polymerase, protecting from +20 to +30. As described in section 1C.3, this fraction

contains a DNA-dependent ATPase activity. Transcription has an energy requirement that can be satisfied by the hydrolysis of either ATP or dATP, generating what is termed an 'activated' transcription complex (Sawadogo & Roeder, 1984). Indeed, addition of dATP causes a dissociation of the complex to give one of the same ability as 4 and loss of TFIIE/F protection. This is explained by the helicase activity of TFIIE/F unwinding the template strand, making it accessible to RNA polymerase. The helicase activity would run off the template, thus explaining the loss of this fraction.

Complex 6. Generated by the addition of NTP's allowing transcriptional elongation, this complex migrates near complex 4 consistent with the model of loss of TFIIE/F. This step is blocked by the addition of α -amanitin (an inhibitor of elongation but not pol II binding), whereas the dATP/ATP step discussed above is not.

The exact roles played by the various transcription factors during the initiation process are as yet unknown, as is the reason why any of the factors remain associated with the RNA polymerase once it enters elongation mode (Flores *et al.*, 1989). However, it appears that at least some of the components of the preinitiation complex remain bound near the initiation site once RNA polymerase has cleared the promoter; minimally USF and TFIID in the case of the AdMLP (Van Dyke *et al.*, 1988). This implies that after the first round of productive initiation, the mechanisms leading to subsequent rounds of transcriptional initiation may be facilitated. However, very little information is available concerning this important process.

1C.5. Upstream Regulatory Sequences

It is clear that the effect of upstream promoter

and enhancer elements is to potentiate the events occurring at the core element, thus facilitating reaction steps that would otherwise be rate limiting.

Upstream activating sequences (UASs) are typically short (8-12 bp) gene-specific motifs that act as binding sites for transcriptional activators (Guarente, 1987; Johnson & McKnight, 1989; Struhl, 1989). These motifs act to stimulate transcription either constitutively, as in the case of CCAAT box elements (Myres *et al.*, 1986) and Sp1 binding sites (Jones & Tijan, 1985), or in response to specific stimuli such as serum stimulation (Triesman, 1985), heat shock (Bienz & Pelham, 1986), viral infection (Goodburn *et al.*, 1985, 1986), exposure to heavy metals (Serfling *et al.*, 1985) and steroids (Renkawitz *et al.*, 1984). UASs can operate in either orientation, but show a decrease in activity with increasing distance from the TATA box (McKnight *et al.*, 1982).

Enhancers are defined by their ability to act over considerable distances both upstream and downstream of the transcriptional start site, and by their independence of orientation in relation to the gene. They are longer than UASs, sometimes comprising hundreds of bases (Edlund *et al.*, 1985), and contain binding sites for many different proteins (Serfling *et al.*, 1985).

Gene specific, and indeed cell specific regulation of transcription occurs largely because different transcriptional activators respond to different sets of regulatory signals.

1C.6. Transcriptional Activators

Transcriptional activators contain distinct domains dedicated to DNA binding at the UAS/enhancer and to the activation of the transcriptional machinery (Brent & Ptashne, 1985; Hope & Struhl, 1986). The DNA-binding domains of activators described so far are of varying classes, termed helix-loop-helix, helix-turn-helix, leucine

zipper and zinc finger (Johnson & McKnight, 1989). There are also several types of activator domains. A majority class, including many yeast activators and HSV Vmw65, has a highly acidic domain enriched in glutamate and aspartate residues (Hope & Struhl, 1986; Ma & Ptashne, 1987; Triezenberg *et al.*, 1988a). Alteration of the sequence of the acidic residues, without changing the overall negative charge, strongly affects activation potential (Giniger & Ptashne, 1987; Hope *et al.*, 1988) thus demonstrating that the physical structure of the acid domain is also critical for function.

Other factors (so far found only in mammals) have nonacidic domains, including the glutamine-rich activation domain of Sp1 (Courey *et al.*, 1989) and the proline-rich CTF/NF-1 (Mermod *et al.*, 1989). It has also been shown that in certain cases, residues around the zinc finger of the DNA-binding domain may play a direct role in the activation of transcription (Kim & Guarente, 1989; Schena *et al.*, 1989). In other cases, the activator is a heteromeric complex containing several protein subunits e.g. AP-1, (Olesen *et al.*, 1987; Gentz *et al.*, 1989; Landschulz *et al.*, 1989; Turner & Tijan, 1989). In some heteromeric complexes, the acidic activation domain is supplied on a discrete polypeptide chain eg Vmw65 (section 1B.3.2), which associate with DNA binding proteins in the nucleus and are thereby directed to particular promoters.

1C.7. Mechanism of Action of Gene-Specific Transcription Factors

The major question which remains essentially unanswered concerns the identity of the primary target(s) of and the exact mechanism of action of the various *trans*-activator proteins. The favoured model for the mechanism of transcriptional activators proposes that activation domains contact a target factor in the basic machinery, thereby looping out the intervening DNA (Ptashne, 1986, 1988). This

contact would activate transcription by stabilizing the binding of the target factor, or by converting the conformation of the bound factor to an active form.

The obvious target in the basal apparatus for such an interaction is TFIID (Horikoshi *et al.*, 1988a, b; Sawadogo & Roeder, 1985b). Consistent with this notion, there is evidence that some transcription factors including ATF and pseudorabies virus IE, assist TFIID binding to DNA (Horikoshi *et al.*, 1988b; Workman *et al.*, 1988). In addition, both GAL4 and ATF factors have been shown to alter qualitatively the interaction of TFIID with the TATA motif, thereby facilitating subsequent interactions with other transcription factors (Horikoshi *et al.*, 1988a, b). However, upstream factors like Sp1 (Schmidt *et al.*, 1989) or CTF/NF-1 (Garcia *et al.*, 1987) do not seem to alter the binding of TFIID. Thus, it is not altogether clear whether gene activators function directly through TFIID or via intermediary fractions that are present in partially purified TFIID fractions.

This latter point was addressed by an intricate line of reasoning by Pugh & Tijan (1990) to define the nature of Sp1 interaction with the basal apparatus. They demonstrated that Sp1 could only stimulate basal level transcription with semi-purified *drosophila* TFIID fractions, and not with cloned TFIID from *drosophila* or yeast. This implied that the interaction between Sp1 and the basal apparatus requires some other component contained only in crude TFIID fractions. This component has been called a coactivator. Further experiments showed that with N-terminal deleted TFIID preparations, induction of basal level transcription was also abolished thus demonstrating that Sp1-induced activation must be mediated via the N-terminal tail TFIID. Such an effect has also been demonstrated for Sp1-induced activation with human TFIID (Peterson *et al.*, 1990).

Another line of evidence has been provided by the

comparison of the effect of the chimeric activator GAL4-Vmw65 on templates that contained only a basal promoter (TATA box), or that had in addition an upstream sequence, either *GAL4-UAS* or *dA:dT-UAS* (Berger *et al.*, 1990). It was shown that when added to a cellular extract, GAL4-Vmw65 induced transcription of the *GAL4-UAS* promoter 100-fold, while its addition to *dA:dT-UAS* promoters, which have no GAL4-Vmw65 binding site almost entirely abolishes transcription. Addition of an oligonucleotide containing a GAL4 binding site restored basal transcription from the *dA:dT UAS* promoter, but cannot restore the induced level. The oligo prevented non-specific binding of GAL4-Vmw65 to DNA, thereby preventing *cis*-inhibition of basal transcription. By contrast, the inhibition of activated transcription from the *dA:dT-UAS* is not determined by GAL4's ability to bind DNA, and is called *trans*-inhibition. It was proposed that GAL4-Vmw65 titrated an 'adaptor' that bridges between the upstream activator and basic transcription machinery. Other experiments suggesting that GAL4-Vmw65 requires a factor that is part of the basal apparatus were reported by Kelleher *et al.*, (1990). Also employing a system in which GAL4-Vmw65 inhibits transcription from the *dA:dT*-promoter, they found that the inhibition could be relieved *in vitro* by a crude yeast nuclear preparation, but not by any known (purified) basal transcription factors including TFIID. Yet the ability of Vmw65 to interact directly with TFIID is suggested by Stringer *et al.*, (1990; see also section 1B.3), however this remains to be proven with purified proteins whether this is the case.

The analysis of the Sp1 and GAL4-Vmw65 systems could be unified if GAL4-Vmw65 is shown to act via an adaptor that recognises the N-terminal region of TFIID. Recent evidence from Lin and Green, (1991) has demonstrated that the yeast acidic transcriptional activator GAL4 stimulates transcription by increasing the

number of functional preinitiation complexes, and that this increase is effected by recruiting the general transcription factor TFIIB to the promoter. They also demonstrated a specific interaction between the acidic activating region and TFIIB, and proposed that TFIIB, or perhaps a very closely associated factor is the direct target of acidic activators in general.

The fact that TFIID can support basal level transcription independently of its ability to interact with the regulatory apparatus is itself a significant advance, yet begs the question of how the factors that bind to the N-terminal domain actually change the transcription complex so as to increase the rate of initiation. This could be purely an internal matter concerning interactions between components of the transcription apparatus, or perhaps it is connected to other changes, for example in the surrounding chromatin.

1D. HSV LATENCY

A major feature of the biology of the herpesviridae is their capacity to establish latent infections, following which they can reactivate to cause episodes of significant, sometimes serious disease. Latency *per se* can be defined as a type of inapparent infection where the viral genome is present, but infectious virus is not produced except during intermittent episodes of reactivation. Despite the enormous advances made recently in understanding the molecular biology of HSV, particularly with respect to the structure and organisation of the virus genome, the molecular events surrounding the establishment and maintenance of and reactivation from latency remain poorly understood.

1D.1. Latency: A Historical Perspective

The phenomenon of recurrent herpes simplex has been recognised for centuries. The concept of a connection between herpetic lesions and the nervous system arose from various clinical observations at the beginning of this century (Cushing, 1905). Involvement of the sensory ganglia emerged from the classical observations of neuropathology of HSV in rabbits by Goodpasture & Teague, (1925) and Goodpasture, (1925) and engendered the view that the nervous system might also be involved in the pathogenesis of herpetic disease in man. These studies lead Goodpasture, (1929), to make what subsequently proved to be a farsighted prediction that HSV can establish a latent infection in the neurons of the trigeminal ganglion. This ganglion supplies sensory nerves to the ophthalmic and facial areas where recurrent herpetic lesions are often observed. This early work was supported by more careful documentation of cutaneous herpetic lesions in man following surgery of the trigeminal tract (Carton & Kilbourne, 1952; Carton, 1953).

Goodpasture's hypothesis was eventually substantiated through direct evidence from experimentation with animal systems, in particular the guinea pig, rabbit and mouse (Stevens & Cook, 1971; Stevens *et al.*, 1972; Knotts *et al.*, 1973; Baringer & Swoveland, 1974; Walz *et al.*, 1974; Scriba, 1975, 1976) and most significantly, from human tissues (Bastian *et al.*, 1972; Baringer & Swoveland, 1973; Plummer, 1973; Warren *et al.*, 1978). Historically, however, incisive studies have proven difficult to design using animal systems, since even under the best experimental conditions, latently infected neurons represent a minority (approximately 1%) of cells in a latently infected sensory ganglion. In addition, neurons are difficult to purify from such tissues. These problems have limited the number of biochemical and molecular biological techniques that can be applied to the systems studied.

1D.2. Animal Models of Latency

HSV can establish latent infections in man, and when inoculated into experimental animals. In all cases, the basic pattern of infection is essentially similar. After initial infection and many rounds of replication at the skin, the following series of events ensues: The virus capsid ascends the nerve axons by retrograde axonal transport to associated somas in sensory ganglia. There, either viral replication with neuronal destruction, or establishment of a latent infection and neuronal survival occurs. In later episodes, the viral genome may reactivate and pass (possibly as a virion in a vacuole) anterograde in axons, crossing from axon to epithelium where a productive infection, and lesions develop.

The first animal latency system to be developed was the mouse footpad model (Stevens & Cook, 1971; 1973a, b; Walz *et al.*, 1974). Following inoculation into the rear footpad, the virus spreads through to the peripheral and

central nervous system, eventually reaching the brain. This develops into an acute infection which peaks at around 5 days post-inoculation (pi) and may eventually lead to temporary posterior paralysis and possibly death. Throughout this period (lasting up to 10 days pi), infectious virus can be recovered from the sciatic nerve, the dorsal root ganglia, the spinal cord and medulla oblongata. After this period, virus can be recovered from the animal by explantation and cocultivation of the lumbrosacral dorsal root ganglia with cell monolayers (Stevens & Cook, 1971); if ganglia are homogenised prior to plating on monolayers, virus cannot be recovered. This series of experiments provided conclusive proof that HSV resides in a latent state in peripheral nervous system ganglia in a non-infectious form. Similar systems of latency in lumbrosacral dorsal root ganglia have been developed for guinea pigs through footpad and vaginal inoculation (Scriba, 1975; 1976).

The other most commonly used systems involve inoculations of the eye of the mouse (Knotts *et al.*, 1974; Walz *et al.*, 1974) and rabbit (Stevens *et al.*, 1972). Following infection of the cornea via scarification or inoculation, HSV establishes a latent infection in the trigeminal ganglion which can also be reactivated by explantation and cocultivation. In addition, latent infections of the trigeminal ganglion and cervical dorsal root ganglion can be achieved through inoculation of the lip and ear respectively (Walz *et al.*, 1975; Hill *et al.*, 1975).

Spontaneous reactivation with recurrence or recrudescence of HSV has been observed with animal systems. Latent virus is not readily reactivated from the mouse; spontaneous recrudescence has only been observed in a small proportion of animals using the ear model developed by Hill *et al.*, (1975). This reactivation was characterised by occurrences of redness and vesicles from which infectious

virus could sometimes be recovered (Hill *et al.*, 1975; Harbour *et al.*, 1981). By contrast however, spontaneous recurrences are common in the guinea pig inoculated vaginally with HSV-2 (Scriba, 1976). Reactivation can be induced in some systems by neurectomy to produce an acute infection in associated ganglia (Walz *et al.*, 1974; Price & Schmitz, 1978). Reactivation can also be induced by exposure to a variety of physical, chemical or systemic stimuli (see section 1D.5.1).

Animal model systems such as the mouse where spontaneous reactivation is rare are suitable for investigation of the requirements for establishment and maintenance of the latent state, whereas other systems where reactivation occurs spontaneously or can be induced are useful for identifying the factors responsible for recurrent herpetic disease.

1D.3. ESTABLISHMENT OF LATENCY

3.1. Sites of Latency

Following initial infection at the periphery, HSV can pass from dermatome to ganglion intra-axonally by retrograde axonal transport (Kristenssen *et al.*, 1971; Hill *et al.*, 1972; Cook & Stevens, 1973; Baringer & Swoveland, 1974; Para *et al.*, 1980). Although the form in which the virus travels has not been firmly established, there is some ultrastructural evidence that it passes as a nucleocapsid (Lycke *et al.*, 1984). This interpretation makes good sense as infection of tissue culture cells involves fusion of the cell membrane and viral envelope followed by entry of the nucleocapsid into the cytoplasm (section 1A.5.1). In the *in vivo* situation, it is likely that the viral envelope is left at the site of entry into the cell, most probably at the intraepithelial nerve

endings. Depending on the site of inoculation, HSV has been shown to establish latency in any sensory or sympathetic nerve ganglion, including autonomic nerve ganglia in mice (Price *et al.*, 1975) and humans (Warren *et al.*, 1978).

During acute HSV-1 infection in trigeminal ganglia, infectious virus is present from 2-8 days pi with a peak on day 4 (Knotts *et al.*, 1974; Rock & Fraser, 1983; Steiner *et al.*, 1988). Infectious virus is not detectable after day 9. Viral RNAs in acutely infected ganglia are detectable from 2-6 days post-infection, with a maximum at 4 days (Spivak & Fraser, 1988a). The presence of viral proteins in trigeminal ganglia during acute infection has been demonstrated by immunostaining and radioimmunoprecipitation at 3 days pi with a peak at approximately 4-5 days pi (Wroblewska *et al.*, 1989). Viral proteins were not detectable from day 6 pi. The reason for this abrupt disappearance was unclear, however, it was proposed that this may have been due to a decrease of viral protein synthesis to below the level of detection, or alternatively, that the antibody response to the infected cells masked antigenic sites (Wroblewska *et al.*, 1988) or the possible removal of infected cells by macrophages.

Subsequent to the period of acute infection, HSV DNA has been demonstrated in tissues of the peripheral nervous system (PNS) (Walz *et al.*, 1976; Puga *et al.*, 1978; Cabrera *et al.*, 1980; Kennedy *et al.*, 1983; Puga *et al.*, 1984) and central nervous system (CNS) (Cabrera *et al.*, 1980; Rock & Fraser, 1983; Stroop *et al.*, 1984) of animals. Latent HSV DNA has also been detected in human brain tissue (Fraser *et al.*, 1981). Although HSV has been demonstrated by explantation and cocultivation to establish latency in the CNS in a small fraction of infected mice and rabbits (Knotts *et al.*, 1973; Plummer *et al.*, 1973; Cook & Stevens, 1976; Cabrera *et al.*, 1980), there appears to be a difference in the efficiency of reactivation from these tissues and those of the PNS. In the study carried out by

Cabrera *et al.* (1980), latent HSV could be recovered by explantation from 95% of trigeminal ganglia, but only 5% of brain tissue explants of the same mice. HSV DNA sequences were, however, detected in the brains of 30% of mice which harboured latent HSV in their trigeminal ganglia, thus suggesting that virus which progresses from the PNS to the CNS is capable of establishing a latent infection in the CNS that cannot be reactivated by the explantation technique.

3.2. Nervous Tissue Cells Harboring Latent HSV

It is now universally accepted that sensory neurons harbour the latent virus, the evidence for which comes from a number of sources: Cook *et al.*, (1974) using their *in vitro* reactivation system showed that the neuron was the cell type in which virus products were initially detected after excision and culture of latently infected ganglia. These results were confirmed and extended by McLennan & Darby (1980) who used *ts* mutants to establish latent infections in mice (which have a core temperature of 38.5°C, the restrictive temperature for mutant growth). They reactivated the virus by peripheral nerve section *in vivo* or by explantation at the restrictive temperature *in vitro*. Since spread of the virus from the site of activation was not possible, and detection of viral antigens was limited to neuronal cells, it was concluded that latent HSV must reside only in the neuron. Kennedy *et al.*, (1983) also detected HSV antigens initially in neurons identified by neuronal-specific antiserum in dissociated cultures from latently infected mouse ganglia. More recent evidence has demonstrated that the latency-associated transcript (LAT; see section 1D.7.2) is detectable only in PNS and CNS neurons (Stevens *et al.*, 1987; Deatly *et al.*, 1988a); their presence has not been detected in any other cell type in latently infected animals.

Although the sensory neurons have been of greatest interest, the fact that many neural tissues have been shown to harbour latent virus (Cook & Stevens, 1976; Warren *et al.*, 1978) suggests that a variety of neuronal cell types can become latently infected.

3.3. Non-Neuronal Sites of Latency

There has been a lot of interest expressed in the possibility of latent infections in non-neuronal sites, particularly of cells at the body surface, and there is evidence from experimental animal systems for establishment of latent infection in both footpads and corneas.

HSV-1 and HSV-2 could be reactivated 12 weeks pi by *in vitro* cultivation of the skin and subcutaneous tissues of the inoculated footpad (Al-Saadi *et al.*, 1983; Clements & Subak-Sharpe, 1983; Subak-Sharpe *et al.*, 1984a, b; Al-Saadi *et al.*, 1988; Clements & Subak-Sharpe, 1988). Virus was not detectable in these tissues immediately at the time of explant. This evidence was strengthened by the demonstration of reactivation of virus from feet which had been denervated 1 week prior to explantation (Clements & Subak-Sharpe, 1988). To decrease the possibility that these animals harboured replicating virus, acycloguanosine was administered in their drinking water beginning 6 weeks after infection. In addition, Al-Saadi *et al.*, (1988) demonstrated that virus could be reactivated from feet of mice which were denervated prior to initial infection, but not from the associated sensory ganglia. *In situ* hybridisation analysis of explanted footpad tissue using HSV-specific probes revealed that the footpad cell types in which HSV RNA first appeared were basal cells of hair follicles, cells of the hair root sheath, in epithelial cells of the sebaceous gland and in cells within the epidermis (Clements & Jamieson, 1989).

Virus has also been reactivated from cultured cells of explanted corneal tissue from rabbits (Cook *et al.*,

1987) and mice (Openshaw, 1983), in cases where infectious virus could not be detected in homogenised tissue fragments. HSV has also been recovered from human corneas removed during corneal transplantation as a result of chronic stromal keratitis (Shimeld *et al.*, 1982; Tullo *et al.*, 1985). However, cultures of skin explants from sites in humans at which HSV recurrences had been observed failed to yield any virus (Rustigian *et al.*, 1966), but recurrent HSV lesions have been observed in patients in areas of skin where the nerve supply had previously been severed (Hoyt & Billson, 1976).

Although it is clear that the PNS ganglionic neurons are the predominant site of latency in human and experimental animals, these results indicate that HSV may reside in a latent state in non-neuronal tissues in mice and rabbits. Any relation of these observations to HSV latency in humans has yet to be established.

1D.4. FACTORS AFFECTING ESTABLISHMENT OF LATENCY

4.1. Viral Factors

The most obvious factor affecting establishment of latency is the ability of the virus to replicate at the periphery or in the sensory ganglia as measured by viral titres in tissue homogenates. Such replication would increase the amount of virus that has the potential to establish a latent infection. Any virus that is unable to replicate at the site of inoculation is less likely to gain access to nerve endings than a virus that is replication competent. A replication-defective virus may be able to enter neurons directly through nerve endings, though such a virus would be unable to leave neurons during reactivation in a cocultivation assay.

Initial studies on the role of HSV-1 replication at the site of inoculation, and in sensory ganglia were based on studies using *ts* mutants (Watson *et al.*, 1980), immune

serum (Klein, 1980), or antiviral drugs (Klein *et al.*, 1979). These studies are, however, treated with caution due to the possibilities that *ts* mutants may leak, and that antiviral drugs and immune serum may not completely block viral replication.

More recent studies using viruses with deletion, insertion and nonsense mutations in essential genes have ensured that viral replication could not occur at any stage of the infection process. Lieb *et al.*, (1989) demonstrated that null mutants with lesions in the essential genes encoding Vmw175 and Vmw63 did not replicate in the eye (the site of inoculation) or ganglion. They also failed to detect any latent virus, either by DNA-blot hybridization or by attempts to rescue the virus from explanted ganglion cultures in the presence of complementing feeder cells or by superinfection with a complementing virus.

Viruses carrying lesions in the IE-1 gene encoding Vmw110 were able to replicate in the eye and establish a latent infection (Lieb *et al.*, 1989; Gordon *et al.*, 1990). These mutants, however, exhibited reduced levels of viral DNA in ganglia relative to wild-type; it is possible that this effect was multiplicity-dependent since defects in the IE-1 gene are only apparent at low multiplicities (see section 1B.4.4). It is likely that low multiplicities of infection are encountered during acute infection and establishment of latency at the ganglion which could therefore explain the low efficiency of Vmw110 mutants in establishing latency.

Steiner *et al.*, (1990) examined the role of Vmw65 during acute and latent infection *in vivo* using the mutant virus *in1814* which contains a 12 bp insertion in the Vmw65 gene and lacks the Vmw65 *trans*-activating function (Ace *et al.*, 1989; see section 1B.3.2). Productive infection of this virus was not detectable in the eyes or trigeminal ganglia of mice, although a low level of replication could not be excluded. Despite its apparent lack of replication,

in1814 was able to establish a latent infection which could reactivate from ganglia with kinetics similar to that of wild-type, thereby demonstrating that Vmw65 is not essential for the establishment of latency.

Various groups have reported that although, ^{independently derived} tk-negative HSV mutants do not replicate in murine sensory ganglia, they do establish latency in these ganglia (Coen *et al.*, 1989; Efsthathiou *et al.*, 1989; Leist *et al.*, 1989; Tenser *et al.*, 1989; Kosz-Vnenchak *et al.*, 1990). Kosz-Vnenchak *et al.*, (1990) used a tk- mutant to examine the initial events leading to latent infection without simultaneous lytic infection in the ganglionic tissue. They demonstrated that in the first few days following their inoculation into mouse corneas, tk- mutant strains of HSV express nuclear LAT RNA in ganglionic neurons without expression of any of a series of viral lytic genes (IE-gene 3; UL30, the DNA polymerase, and the late gene encoding gC). Only a very limited level of expression of IE-genes 1 and 2 were detectable using *in situ* hybridization. The observation that a defect in tk, an early gene product, led to decreased levels of IE gene products was surprising. The use of reduced levels of wt virus inoculation resulting in reduced ganglionic virus levels did not mimic the situation observed with the tk- viruses, thus demonstrating that the tk- phenotype was due to some kind of novel behaviour of the mutant virus, and not a result of less virus reaching the ganglion. This demonstrated that expression of immediate-early and other lytic genes is actually reduced in neurons during establishment of latent infection.

Another HSV-mutant that has been investigated is a virus with a deletion in the gene encoding the large subunit of ribonucleotide reductase. Although it can replicate in many cell types in culture (Goldstein & Weller, 1988a), it is highly defective for replication in mouse cells at 38.5° (Jacobsen *et al.*, 1989). Following corneal inoculation, this mutant replicated very poorly in

the eye, achieving barely detectable titres. It also failed to achieve detectable titres in trigeminal ganglia during the four days post-inoculation, or to reactivate from these ganglia upon explant 30 days post-inoculation (Jacobsen *et al.*, 1989).

Recently, Katz *et al.*, (1990) using polymerase chain reaction assays to detect viral DNA have shown that viral genomes can associate with ganglia despite lack of any replication at the site of inoculation. The amount of HSV DNA detectable in the ganglion showed a rough, but not absolute correlation with the ability of the virus to undergo productive replication. While wt (KOS) virus DNA was found at 0.5 copies per cell (5×10^5 per ganglion), tk- and ribonucleotide reductase- mutants were detected at 0.01 copies per cell (10^4 per ganglion). The finding that HSV DNA levels in mice infected with tk- and rr- viruses were similar was surprising since, unlike rr- mutants, tk- mutants replicate to wild-type levels in the mouse eye. Interestingly, viral DNA could also be detected in ganglia of mice infected with non-replicating HSV mutants with lesions in IE-genes 2 and 3, and in UL30, (encoding the DNA polymerase), at 0.0001 copies per cell (10^2 per ganglion). These mutants must have gained access to ganglia by budding directly into the corresponding nerve-endings upon inoculation and demonstrated that mutants which are severely restricted for productive virus replication and gene expression can establish latency.

Despite a large number of studies there is no evidence for any HSV mutants which fail to establish latency. These include mutants in IE-gene 5, glycoproteins G and E, the HSV protein kinase and the genes US11 (21K DNA binding protein) and US12 (Meignier *et al.*, 1988). It therefore appears that establishment of latency does not require any virus gene expression.

Several hypotheses have now been advanced to explain the non-permissive infection of neurons by HSV

which leads to latent infection:

- i) lack of necessary cellular transcription factors for viral IE-gene expression in certain neurons (Roizman & Sears, 1987).
- ii) lack of transport of the virion *trans*-inducing factor to the neuron nucleus, leading to reduced level of viral IE-gene products (Roizman & Sears, 1987).
- iii) presence of an inhibitor of IE-gene expression in neuronal cells (Kemp *et al.*, 1990).
- iv) dominance of viral negative regulatory gene products in neurons leading to reduced level of early or late gene products (O'Hare & Hayward, 1985b; Su & Knipe, 1989).

All studies carried out so far suggest that the establishment of HSV latency does not require the lytic pathways of gene expression, and that viral replication itself merely permits an increase in the number of viral genomes that can gain access to neuronal nuclei. It is possible that as yet unidentified viral regulatory proteins promote the latency pathway, however, all available results suggest that establishment of latency is a passive process that is governed by neuronal factors rather than by any *de novo* synthesised viral gene product.

4.2. Neuronal Factors Affecting Latency

The neuronal cell is long-lived and non-mitotic and cellular DNA synthesis occurs only as a consequence of DNA repair mechanisms. The relatively quiescent state of the neuronal genome may therefore have a role in permitting HSV to reside in a similarly inactive state. Most work examining the interaction of HSV with cells of neuronal origin has been through the use of neuronal cell lines, since the amount of material available from primary cultures of sensory neurons have proven insufficient for any informative studies.

Vahlne & Lycke (1977, 1978) demonstrated that the

neuronal cell line C1300 is non-permissive for lytic infection with HSV. This effect was shown by nuclear run-on assays (Kemp & Latchman, 1989) to be mediated by a failure to transcribe the viral IE genes following infection, and that this block could be relieved by pretreating the cells with sodium butyrate which is known to increase their permissivity to HSV infection (Ash, 1986). Kemp *et al.*, (1990) subsequently demonstrated that this transcriptional repression is mediated by the binding of a repressor factor to the octamer-related TAATGARAT motif (see section 1B.3.1). This repressor activity was specific to cells of neuronal origin (being absent in a range of permissive non-neuronal cells) and is also able to repress a range of cellular octamer-containing promoters introduced into C1300 cells; the repression could be relieved by mutagenesis or deletion of the octamer element.

Although the specific nature of this octamer-binding protein has not been identified, it is interesting to note that several octamer-binding proteins other than the ubiquitous oct-1 and B-cell specific oct-2 have been identified in other cell types, most notably in embryonal carcinoma cells (Lenardo *et al.*, 1989; Schöler *et al.*, 1989) which like C1300 cells are non-permissive for HSV (Bell *et al.*, 1987). He *et al.*, (1990), when examining expression of a series of POU-homoeodomain genes (including oct-1 and oct-2) in embryos and adults by *in situ* hybridisation found that oct-1 gene expression in the adult rat nervous system was highly restricted, being detectable only in certain regions of the brain; oct-1 expression could not be detected in any of the sensory or trigeminal ganglia. Instead, they found low levels of a novel POU-domain gene (termed Tst-1) expressed in sensory ganglia and low levels of two other novel POU-domain proteins (termed Brn-1 and Brn-2) in the trigeminal ganglia.

During the establishment of latency, the lack of

transport of Vmw65 to the neuron nucleus, coupled to the lack of oct-1 in neuronal cells probably leads to the suppression of IE gene expression and therefore the suppression of lytic infection. It would be intriguing to propose that the novel POU-domain proteins found in neuronal cells, like oct-2, are unable to support Vmw65-mediated *trans*-induction of immediate-early gene expression, and may indeed repress IE gene expression by binding the TAATGARAT motif as proposed by Kemp *et al.* (1990).

4.3. Immunological Factors Affecting Latency

Initial infection by HSV-1 is usually followed by the appearance of a protective specific immune response which includes neutralizing antibodies directed against glycoproteins present in the viral envelope (Carter *et al.*, 1982) and cytotoxic T-cells which, at least in the murine model, are directed against structural glycoproteins in the virion envelope and in the infected cell (Carter *et al.*, 1982; Glorioso *et al.*, 1985; Martin *et al.*, 1988) and non-structural proteins, predominantly the products of the immediate-early genes (Martin *et al.*, 1988).

The role of the immune response in protection against the establishment of a latent infection is not very clearly understood. It has been demonstrated though that it is difficult to establish latent HSV infection in animals which have been either previously infected or immunized against HSV infection (Walz *et al.*, 1976; Cremer *et al.*, 1985; Rooney *et al.*, 1988). Neutralizing antibody (Walz *et al.*, 1976; Cremer *et al.*, 1985; Rooney *et al.*, 1988), infiltrating B-lymphocytes (Cook & Stevens, 1983) and T-lymphocytes (Nash *et al.*, 1987) have all been implicated as being important in preventing infection of neurons or in restricting HSV multiplication once the virus is in the ganglia. It has been demonstrated that the adoptive transfer of HSV-specific cytotoxic T-lymphocytes (CTL's) to

recipient animals immediately before infection in the footpad significantly decreases the levels of infectious virus able to be recovered from the footpad (Larsen *et al.*, 1984; Rouse *et al.*, 1985; Bonneau & Jennings, 1989). This treatment also reduced the levels of HSV able to be reactivated from latency, thus suggesting that CTL's may play a role in the control of HSV infection and restriction of HSV multiplication in ganglia.

Although there is clear evidence that prevention of HSV replication by the immune response can greatly reduce reactivatable latent infections, it has been suggested that this is due to decreasing the number of virus genomes that are capable of establishing latent infections (Katz *et al.*, 1990). These observations have lead to speculation that vaccine strategies targeted against lytic functions of the virus may be unable to prevent HSV latency completely.

1D.5. MAINTENANCE AND REACTIVATION FROM LATENCY

5.1. General Considerations

Reactivation of latent HSV in humans can be induced by a number of stimuli including exposure to ultraviolet light, fever, stress and hormonal changes (Hill, 1985). Reactivation can also be induced in a number of animal model latency systems by exposure of their periphery to a variety of physical and chemical stimuli, including exposure to uv light (Blyth *et al.*, 1976), repeated applications of cellophane tape (Hill *et al.*, 1983) or chemicals such as dimethylsulphoxide (DMSO) and xylene (Harbour *et al.*, 1983).

Such evidence has led to a number of theories pertaining to the nature of reactivation, the most favoured being the 'ganglion and skin trigger' theory (Hill, 1985). This proposes that following a peripheral stimulus, reactivation of the latent infection in the ganglion results in the production of infectious virus which

proceeds along the associated nerves to the periphery where a recurrent lesion may occur. It has also been suggested that whenever a recurrent lesion occurs, any infectious virus produced may travel up the peripheral nerve again, and latently infect more neurons; the so-called 'round-trip' theory (Klein, 1976). The nature of the message that is stimulated by the various reactivation-inducing treatments is not clear. UV light is known to produce many changes in the skin including damage to epithelial cells and their replication (Epstein *et al.*, 1971), the release of prostaglandins (Harbour *et al.*, 1983) and the reduction of ATPase staining of Langerhans cells (Bergstresser *et al.*, 1980). In rabbits, it causes epithelial loss and swelling in the cornea (Riley *et al.*, 1987) and killing of keratinocytes (Ringvold & Davanger, 1985). It is not clear, though, how these observations relate to the reactivation of HSV. It is possible that reactivation may arise from the direct effect of uv light on the peripheral nerve endings. A common factor between the different reactivation stimuli appears to be their ability to induce inflammation of the skin (Harbour *et al.*, 1983); the physiological changes which mediate this response could also affect the neuronal cell body.

Reactivation may also be related to the metabolic changes in neurons associated with repair of damage to nerves or nerve endings (Price & Schmitz, 1979). In this respect, it is likely that the physical and chemical stimuli to the skin all produce alterations or damage in the membranes of the nerve endings and thereby cause such metabolic changes in the ganglion.

HSV reactivation can also be induced by peripheral nerve section (neurectomy) as occurs, for instance, during explantation of nervous tissues. This process causes a profound alteration in the metabolism of neurons, possibly induced by the loss of a trophic factor (eg nerve growth factor) from the periphery. These changes include an

increase in the general level of transcription, protein synthesis and DNA synthesis. Reactivation of HSV from latently infected ganglia can be enhanced by co-cultivation of intact or dissociated ganglia on permissive cell monolayers (Knotts *et al.*, 1973; Wohlenberg *et al.*, 1979; Harbour *et al.*, 1981), by heterotypic superinfection of ganglia (Thomas *et al.*, 1985) or by exposure of ganglia to demethylating agents (Bernstein & Kappes, 1988; Stephanopoulos *et al.*, 1988).

DNA methylation at the dinucleotide 5'-CG-3' has been correlated with transcriptional inactivity and appears to be an important factor concerning regulation of eukaryotic gene expression. In general, hypomethylation is a necessary, but not sufficient precondition of gene transcription. In several *in vitro* viral systems, including adenoviruses, herpesviruses and retroviruses, transcriptionally active or replicating viral genomes are consistently hypomethylated, or specific inactive regions of viral genomes are readily heavily methylated (Doerfler, 1981; Yousoufian *et al.*, 1982; Szyf *et al.*, 1985). The observations that demethylation agents such as HMBA (Bernstein & Kappes, 1988; Stephanopolous *et al.*, 1988) and 5-azacytidine which functions to prevent methylation of DNA all induce reactivation of HSV from neural tissues have implied that the latent HSV genome was maintained in a transcriptionally quiescent state in neurons by extensive methylation. However, Dressler *et al.* (1987) subsequently demonstrated with methylation-sensitive restriction endonucleases that latent HSV-1 DNA was not extensively methylated *in vivo*. Consequently, reactivation of latent HSV using demethylation agents probably occurs indirectly by converting the host cell to a permissive state for HSV replication via cellular gene activation.

5.2. Viral Factors Affecting Reactivation

These factors have been investigated using the same

virus deletion and insertion mutants described in section 1D.4.1 when examining factors affecting establishment of latency. Steiner *et al.* (1990) demonstrated that HSV *in1814* (the Vmw65 insertion mutant) reactivated upon explantation as efficiently and rapidly as wt. This suggested that reactivation may be caused by the induction of cellular factors which complement the *trans*-inducing activity of Vmw65 and lead to expression of IE genes and viral replication. Since Vmw65 is not expressed during latency (Deatly *et al.*, 1987; 1988; Spivak & Fraser, 1987; Stevens *et al.*, 1987), and since during reactivation, IE genes would be expressed prior to Vmw65 (a late gene), Vmw65 is therefore unlikely to play any role in the initial events of the reactivation process.

The role of the HSV IE genes in reactivation has been examined by Leib *et al.* (1989). They showed that, as anticipated, null mutants in IE genes 2 and 3 (encoding Vmw63 and Vmw175 respectively) failed to reactivate from latent infections. They also showed that, despite their replication competence, HSV strain KOS-derived Vmw110 deletion mutants *dlx0.7* and *dlx3.1* failed to reactivate from standard explant cultures of latently infected ganglia. The *dlx3.1* virus could, however, be reactivated by the addition of DMSO to the medium of explant cultures. Both mutants could be reactivated following superinfection with a complementing virus. By contrast, the HSV strain 17+ derived IE-1 deletion mutant *d11403* does reactivate in the absence of DMSO (Clements & Stow, 1989; Leib *et al.*, 1989), therefore demonstrating that Vmw110 may not be essential for reactivation. It is possible that the observed differences in reactivation phenotype of the KOS and 17+ derived mutants are strain specific which may allow 17+, but not KOS to reactivate in the absence of Vmw110. Indeed, major differences in the neurovirulence of strains 17 and KOS have been noted (Thompson *et al.*, 1986). Alternatively, the reactivation phenotypes may arise from the differences

between the deletions in Vmw110. Mutant *dlx3.1* has a 2965 bp deletion, including the transcriptional start site, and should not express any form of the IE-1 transcript or polypeptide, whereas, *dlx0.7* has a 798 bp deletion and would specify an amino-terminal 19 amino acid polypeptide encoded by the first exon. The 3' limit of the *dlx0.7* deletion lies in the second intron, such that the third exon is read out of frame. The *dl1403* mutant has an internal 2117 bp deletion and could specify the amino-terminal 105 amino acids encoded by the first, and part of the second intron. The carboxy-terminus of the third exon which is out of frame. It is possible that the 86 amino acids specified by the undeleted portion of the second exon of *dl1403*, and not found in the other two deletions, are sufficient to render this mutant latency competent *in vivo*. By contrast, the 19 amino acids specified by the first exon of *dlx0.7* are apparently not sufficient to give this mutant the ability to establish or reactivate from latency effectively. The reason for the difference in latency competence between *dlx0.7* and *dlx3.1* is not clear. The possibility that other differences had occurred in these viruses during their construction cannot be dismissed, and therefore demonstrates the importance of characterizing rescued versions of such mutants when investigating their phenotypes. It is also possible that these differences may be a consequence of the deletion of the corresponding regions of the LAT transcript (see section 1D.7.2). For example, the least latency-competent virus, *dlx0.7*, is the only mutant whose deletion did not cover the mapped 3' end of the 2 kb LAT transcript.

Further studies of *dlx3.1* (Gordon *et al.*, 1990) revealed that host species is also a factor in determining the ability to reactivate upon cocultivation. Reactivation could not be observed from CD-1 and A/J mice, as opposed to 30% of Balb/c mice and 22% of NZ rabbits. They also showed that *dlx3.1* could also reactivate and shed spontaneously

from NZ rabbits. Interestingly, the wt KOS strain which could be reactivated by cocultivation from 88% of rabbits, did not shed spontaneously, nor could it be induced to shed by iontophoresis of adrenergic agents.

Other replication competent mutants that fail to reactivate from explanted ganglia in the mouse system include thymidine kinase negative (tk⁻) mutants (Coen *et al.*, 1989; Efsthathiou *et al.*, 1989; Leist *et al.*, 1989; Tenser *et al.*, 1989) and ribonucleotide reductase negative (rr⁻) viruses (Jacobson *et al.*, 1989). The tk⁻ mutants can establish and maintain latency as evidenced by expression of latency-associated transcripts (Coen *et al.*, 1989; Leist *et al.*, 1989; Tenser *et al.*, 1989) and by their ability to be rescued following superinfection of dissociated ganglia by a complementing virus (Coen *et al.*, 1989; Efsthathiou *et al.*, 1989). Both tk and rr mutant genomes could be detected in latently infected ganglia by PCR (Katz *et al.*, 1990). As both tk⁻ and rr⁻ mutants are also severely impaired for acute ganglionic replication, their requirement for reactivation could be explained if there is a requirement for a sufficient pool of their enzymatic products in nucleotide metabolism, in each of these settings to permit viral replication. Neurons, being non-mitotic, are likely to harbour low levels of deoxyribonucleotides, therefore possibly explaining why HSV has enzymes dedicated to nucleotide metabolism.

5.3. Cellular Factors and Reactivation

The observation that various treatments can enhance reactivation of latent HSV from latently-infected explanted ganglia, and in particular the reactivation of Vmw110 deleted *dlx3* by DMSO (Leib *et al.*, 1989), has suggested a possible role for cellular factors involved in the early stages of reactivation. The mode of action of DMSO is unknown, but it has been shown to affect a number of cellular processes (De La Torre, 1983). It would be

interesting to speculate that DMSO may induce reactivation through induction of specific cellular genes. Indeed, host cellular trophic factors such as nerve growth factor (Wilcox & Johnson, 1987) and prostaglandins (Kurane *et al.*, 1984) have been shown to affect the reactivation of HSV-1 from murine ganglia, presumably by perturbation of the host cell. In this respect, it is interesting to note that HIV-1 gene expression in monocytes has been demonstrated to be activated following induction of the transcription factor NF- κ B (Griffin *et al.*, 1989).

1D.6. THE STATE OF THE LATENT VIRUS GENOME

Analysis of the physical structure of HSV DNA from latently infected neurological tissue may provide useful information concerning the molecular mechanisms involved in latency. Initial studies by Rock & Fraser (1983) indicated that viral DNA found in the murine trigeminal ganglion and brain stem was in a different state to that observed during acute infection, and was also different to that present in virions. These genomes lacked detectable termini, suggesting that they could be integrated into multiple sites in the host cell genome or were present in some extrachromosomal form. In the latter case, they could exist as multimeric linear or circular molecules or monomeric circles. These results were confirmed by Efsthathiou *et al.* (1986) who showed that virus genomes with similar properties were present in latently infected human trigeminal ganglia, and that all four DNA isomers could be found in latently infected tissues. Rock & Fraser (1985) subsequently demonstrated that the genomic termini of latent viral DNA were covalently linked; a result compatible with single copy plasmids or concatameric circular or linear molecules.

Mellerick & Fraser (1987), by subjecting the DNA present in murine brain stems to buoyant density gradient

centrifugation, succeeded in separating the viral DNA from the cellular DNA, thus indicating that the viral DNA existed in an extrachromosomal state. This suggested that the most likely physical form of the viral DNA would be a circular episome. Finally, Deshmane & Fraser (1989) indicated that latent HSV-1 DNA has a nucleosomal structure similar to that of cellular chromatin, as determined by micrococcal nuclease digestion. All of the regions, including the transcriptionally active region of the genome were found to be associated with nucleosomes. This contrasts with HSV DNA found during lytic infection of tissue culture cells (Leibach & Summers, 1980; Seal *et al.*, 1988) and during acute infection of mice (Muggeridge & Fraser, 1986) where a small fraction (10-15%) of viral DNA is associated with nucleosomes. It is possible that this chromatin formation may be essential for the long term stability of viral DNA in the cell nucleus.

An accurate determination of the number of genomes per latently infected cell has proven difficult. Estimates using quantitative blot hybridizations include values of 0.01 to 0.4 genomes per cell in human trigeminal ganglia (Efsthathiou *et al.*, 1986) and 0.015 to 0.1 copy per cell in various murine neural tissue (Cabrera *et al.*, 1980; Rock & Fraser, 1983; Efsthathiou *et al.*, 1986). Quantitative polymerase chain reaction analysis of individual latently infected murine ganglia indicated values of between 0.01-1 copy per cell (Katz *et al.*, 1990). Assuming that one of these values represents the number of genomes actually present in a tissue, and that most of the genomes are non-defective, the actual number of per latently infected neuron would be much larger. This is because neurons constitute only a minor proportion of the cells populating neural tissues (about 10% of the cells in spinal ganglia) and because not all of the neurons in a latently infected ganglion would be expected to harbour viral DNA. Taking these factors into consideration, and scoring for neurons

expressing the latency-associated transcript (assuming that all latently infected genomes express LAT), Stevens (1989) calculated that there may be up to 20 copies of viral DNA per latently infected neuron.

Interestingly, *in situ* hybridisation, a technique which in other systems has been sufficient to detect viral genomes of less complexity and present at only one copy per latently infected cell (hepatitis B virus in hepatocytes; Blum *et al.*, 1983) has not been able to detect HSV DNA in latently infected neurons. The reason for this is not apparent; the DNA is possibly present in a complex that is not accessible when current methods of section preparation are used.

1D.7. LATENT HSV GENE EXPRESSION

7.1. The Search for Latent Viral Gene Expression

Various attempts have been made to define the nature of HSV gene expression by latent viral genomes, and have employed such techniques as DNA/RNA hybridization and immunological procedures for the detection of virus-specific proteins. There have been a few reports of detection of viral proteins during latency in animals (Yamamoto *et al.*, 1977; Green *et al.*, 1981) and during virus reactivation in humans (Vaifa *et al.*, 1988), however, such reports have proven difficult to corroborate. Green *et al.* (1981) using immunofluorescence, detected Vmw175 in latently infected trigeminal ganglia of rabbits, however, further studies failed to substantiate this (PePOSE, 1986) and indeed, the transcript for this protein does not appear to be expressed during latency (Deatly *et al.*, 1987, 1988; Stevens *et al.*, 1987). It is possible that the detection of Vmw175 was due to spontaneous reactivation in rabbits. Similarly, Yamamoto *et al.* (1977) reported the presence of enzymatically active HSV-specific tk in the sensory ganglia of latently infected mice, however, as with Vmw175, tk mRNA

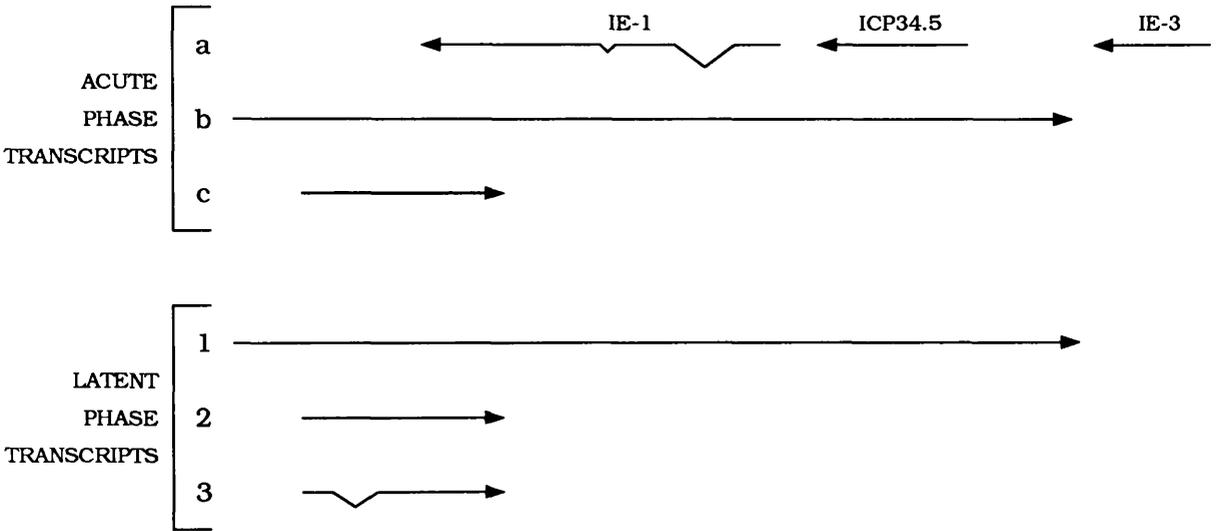
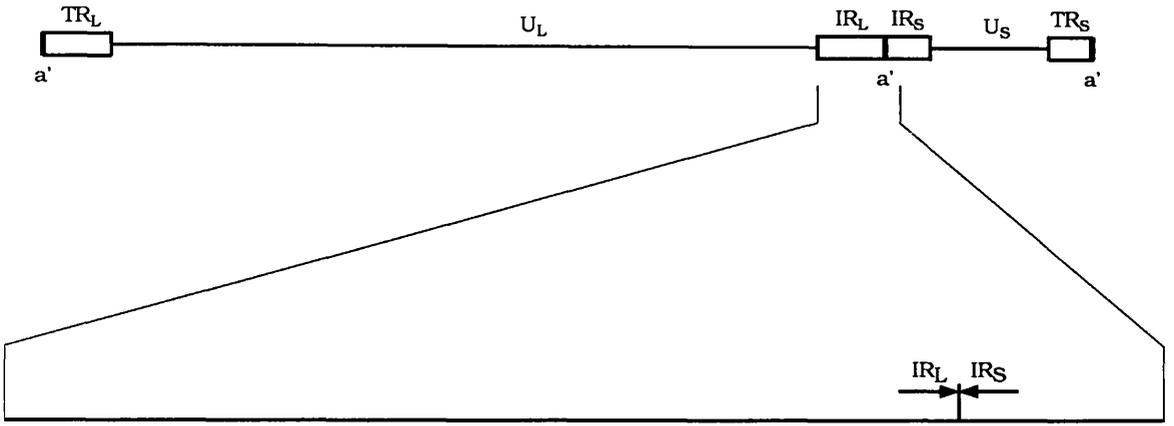
expression cannot be detected in latently infected mouse, rabbit or human ganglia by *in situ* hybridization analysis (Deatly *et al.*, 1987; Puga & Notkins, 1987; Rock *et al.*, 1987a; Stevens *et al.*, 1987b; Steiner *et al.*, 1988).

7.2. The Latency-Associated Transcript

A major development in the field of latency was the discovery by Stevens *et al.* (1987) that a unique transcript (now termed the latency-associated transcript; LAT) is synthesised in latently infected murine sensory neurons. This pattern of transcription is now known to take place in latently infected sensory neurons of mice (Puga & Notkins, 1987; Spivak & Fraser, 1987; Stevens *et al.*, 1987), rabbits (Rock *et al.*, 1987b; Wagner *et al.*, 1988b) and most importantly, in humans (Croen *et al.*, 1987; Gordon *et al.*, 1988; Steiner *et al.*, 1988; Stevens *et al.*, 1988; Wechsler *et al.*, 1988a). *In situ* hybridization has demonstrated that this transcription unit maps to the long repeats of the HSV genome (Figure 5) and is localized exclusively to the nucleus.

The number of transcripts, their sizes and relative proportion to each other differs slightly between laboratories, and depends upon virus strain or experimental system utilized. The major transcript is approximately 2.0 kb (Spivak & Fraser, 1987; Wechsler *et al.*, 1988b) to 2.2 kb (Wagner *et al.*, 1988b) in length and consists of from 50 to 90% of the total. The next most abundant (10 to 50% of the total) is a spliced derivative of the first and is about 1.5 kb long (Spivak & Fraser, 1987; Wagner *et al.*, 1988b; Wechsler *et al.*, 1988b). In addition to these two transcripts, a third, very minor transcript of 1.45 kb has been described by Spivak & Fraser, (1988a) and probably represents yet another spliced derivative of the primary transcript. As a group, these transcripts are minimally polyadenylated (Spivak & Fraser, 1987) or nonpolyadenylated (Wagner *et al.*, 1988b).

Figure 5. Transcription patterns of the HSV-1 internal repeat region in productively and latently infected cells. A diagram of the physical structure of the viral genome is represented at the top of the panel. Although not indicated, transcripts originating in IR_L and IR_S are also encoded by TR_L and TR_S. In latently infected cells, only transcripts originating from the repeat regions can be detected (U_S and U_L are silent). During acute infection of tissue culture cells, the following transcripts can be seen: a) transcripts known to be expressed only during lytic infection; b) polyadenylated transcripts (m-LATs) 8.3 kb in length, and c) unspliced, non-polyadenylated 2 kb LAT transcripts. In latently infected sensory ganglia, in addition to m-LAT (1) and the 2 kb LAT (2), the accumulation of a second (spliced) derivative of the 2 kb LAT (3) is observed.



The most intriguing aspect of the LAT RNAs is that they are all transcribed from the DNA strand opposite (antisense to) that encoding the transcript for the immediate-early polypeptide Vmw110. The major LAT transcripts all begin approximately 1210 bp downstream of the IE-1 mRNA. The 3' end of LAT overlaps the 3' end of IE-1 by approximately 1000 nucleotides (Wechsler *et al.* 1988b). There are two significant open reading frames which are not interrupted by the splice in the two major species of RNA (Wagner *et al.*, 1988b; Wechsler *et al.*, 1988b), but there is no evidence that a protein is encoded by these species. Indeed, sequence analysis of the LAT gene and its open reading frames by Perry & McGeoch (1988) revealed that the codon usage characteristics of the LAT ORFs were atypical for HSV and they concluded that it was unlikely that LAT encoded any proteins.

Small amounts of the unspliced transcript (also non-polyadenylated) are found in lytically infected tissue culture cells (Krause *et al.*, 1988; Spivak & Fraser, 1987; Stevens *et al.*, 1987; Wagner *et al.*, 1988b). Analysis of the kinetics of their transcription has revealed that LAT does not appear to fit any of the typical kinetic classes of HSV transcripts previously described (Spivak & Fraser, 1988a). A rough calculation by Wagner *et al.* (1988a) has indicated that the LATs as a group are present at 2×10^4 to 5×10^4 molecules per latently infected murine neuron.

More recent evidence has suggested that the LAT transcription pattern is considerably more extensive than was previously thought. Additional weak hybridisation signals were detected by *in situ* hybridization which corresponded to transcription from DNA fragments adjacent to either side of the 2 kb LAT domain (Dobson *et al.*, 1989; Mitchell *et al.*, 1990; Zwaagstra *et al.*, 1990). A combination of northern blot analysis (Zwaagstra *et al.*, 1990) and *in situ* analysis (Dobson *et al.*, 1989; Mitchell *et al.*, 1990; Zwaagstra *et al.*, 1990) demonstrated the

presence of an 8.3 kb transcript in latently infected neurons and lytically infected tissue culture cells. This RNA, termed the minor latency-associated transcript (m-LAT) was found to be the same sense as LAT, and extends from 660 bp upstream from the 2 kb LAT to the first consensus polyadenylation signal in the short terminal repeat just downstream from the IE-3 gene. The 5' end of the m-LAT was mapped by primer extension of RNA of transfected cells to approximately 28 bp downstream from the first T of the TATA box (Zwaagstra *et al.*, 1990) of the putative LAT promoter (Wechsler *et al.*, 1988b, 1989). Although not proven, it is likely that the 2 kb and 1.5 kb LATs are derived from the 8.3 kb LAT. Since mutants lacking the immediate region around the LAT promoter do not produce either the 8.3 kb LAT or the 2 kb and 1.5 kb LATs (Dobson *et al.*, 1989; Mitchell *et al.*, 1990b), it is possible that the 2 kb LAT is derived from the 8.3 kb LAT and that the other LAT species are derived by splicing of the 2 kb LAT. This raises the possibility that the 1.5-2.0 kb LATs are processing products (Dobson *et al.*, 1989) or stable introns derived from the the larger transcript (Dobson *et al.*, 1989; Mitchell *et al.*, 1990b). Indeed, a recent study by Devi-Rao *et al.*, (1991) has demonstrated that LAT is uncapped, and that its 3' end maps to a canonical splice acceptor site 1,950 bases 3' of its 5' end. They also identified a major species of poly(A)⁺ LAT equivalent to m-LAT. The idea that the 2 kb LAT is a stable intron has been confirmed by Farrell *et al.* (1991) who showed that when a 2.4 kb HSV-1 fragment encompassing the 2 kb LAT is cloned into the *lacZ* gene in pCH110, LAT was correctly spliced out giving rise to a processed β -gal transcript and 2 kb LAT intron. All of these data have demonstrated that all latent phase transcription is via a single large primary transcript which is spliced to yield an abundant poly(A)-species; the LAT intron. The spliced polyadenylated product of this splicing reaction has, however, remained

undetected.

7.3. A Function for LAT?

Since the discovery of the LATs, a great deal of effort has been employed in order to elucidate their function(s). It is possible that these transcripts could function in any of the stages of establishment of, maintenance of or reactivation from the latent state. To date, the majority of work dealing with this subject has focussed on the latency characteristics of various mutants with deletions or insertions in the LAT gene.

Javier *et al.* (1988) reported that an HSV-1xHSV-2 recombinant (X10-13) which contains a small HSV-2 insert and a small deletion in the LAT sequences does not express LATs but ^{can} establish latency after footpad inoculation. Since both X10-13 and wt virus could be recovered from lumbar ganglia of latently infected mice with equal frequency at similar times after explantation, it was concluded that the LATs are dispensable for the establishment of latent infections. Similarly, Sederati *et al.* (1989) using a LAT-KOS derivative (KOS 8117) and its LAT⁺ rescued derivative (KOS D362) found that both viruses latently infected the same number of neurons and contained the same copy number of genomes per infected cell. The amount of viral DNA did not differ over an 11 month period and therefore suggested that LAT has no effect on the establishment or maintenance of latency. They did notice, however, that the expression of lytic phase transcription of LAT- KOS 8117 during acute infection of ganglia was delayed when compared to wt. An HSV-1 insertion mutant, RH142 (Ho & Mocarski, 1989), generated by placing the *lacZ* gene under LAT promoter control did not express LAT in latently or productively infected cells, however β -galactosidase was readily detected in sensory ganglia, but not Vero cells. This virus was able to establish and reactivate from the mouse eye mode latency system with the kinetics and efficiency of wt

virus.

By contrast, other studies, while supporting a non-essential role in establishment and maintenance of latent infection, have suggested a role for the LATs in the reactivation process. HSV-1 variant 1704 which possesses a 3.8 kb deletion in IR_L and adjacent unique DNA sequences, and a 1.2 kb deletion in TR_L (Maclean & Brown, 1987a), does not express LATs (Steiner *et al.*, 1989) or m-LATs (Mitchell *et al.*, 1990) in tissue culture or latent infection of mice. Although 1704 could establish latent infections in murine trigeminal ganglia, explant reactivation of the virus was significantly slower (31 days for 8/9 mice) than wt (100% within 7 days) (Steiner *et al.*, 1989). The survival rate of 1704 infection (79%) was also significantly better than wt (38%). In addition, Leib *et al.* (1989) have reported that a KOS-derived LAT- deletion mutant (*dLAT8.1*) reactivates from explants less efficiently than its LAT⁺ parent. Interestingly, Hill *et al.* (1990), also using HSV X10-13 (see above) and its rescued LAT⁺-derivative XC-20 showed that both viruses replicated in the rabbit eye and established latency with equal efficiency. Similar percentages of trigeminal ganglia latently infected with either virus produced HSV when cultivated *in vitro*, and a small proportion of rabbits latently infected with either virus spontaneously shed virus in their tear films episodically during the latent phase of infection. However, when reactivation was induced by iontophoresis of epinephrine it was found that while rabbits infected with XC-20 reactivated with efficiencies similar to wt (18/20), the efficiency of X10-13 reactivation (3/20) was significantly reduced.

The reason for the effect of LAT expression on the *in vitro* recovery of virus not being as marked as observed for induced reactivation *in vivo* is not clear. Explanation and maintenance of latently infected neurons *in vitro* for virus recovery subjects the cells to a number of insults,

and it is possible that this may not fully reflect the processes occurring in the animal. *In vitro* reactivation of virus usually results in neuronal cell death, however, *in vivo* reactivation is more likely to require the survival of neurons, and that this could be the result of an alteration or attenuation of the normal lytic phase of viral replication; such conditions may not be obtainable during *in vitro* explantation. In this respect, it is interesting to note that Hill *et al.* (1987) reported that 10 strains of HSV demonstrated the same frequency of recovery from cultured latently infected trigeminal ganglia irrespective of whether the eyes had or had not shed virus spontaneously.

From a direct functional point of view, all available evidence indicates that LAT is in some way involved during reactivation from latency (Leib *et al.*, 1989; Steiner *et al.*, 1989; Hill *et al.*, 1990). Various hypotheses have been forwarded for its role. These include that the transcripts could function physically by somehow "scaffolding" latent viral DNA and making the genome available for reactivation or other processes related to reactivation (Hill *et al.*, 1990). A second model involves some processed form of LAT which could function as an mRNA encoding a protein which facilitates reactivation (Stevens *et al.*, 1987). This would predict that inactivation of one or more reading frames would interfere with reactivation. Indeed, a recent report by Doerig *et al.* (1991) has reported the detection of a 80kDa antigen with an antibody raised against a region of the larger of the two ORF's encoded by LAT in neuronal cell cultures latently infected with HSV-1. Finally, various data (Leib *et al.*, 1989; Steiner *et al.*, 1989; Block *et al.*, 1990; Hill *et al.*, 1990) has ruled out an earlier suggestion (Stevens *et al.*, 1987), that LAT might act directly to facilitate latency by suppressing gene activation by Vmw110 through antisense inhibition of IE-1 gene transcription. If this were the

case, LAT deletion mutants might be expected to reactivate efficiently, or would not establish latent infections at all since there would be no LAT to suppress Vmw110 gene transcription. It cannot be ruled out, however, that LAT might interact with the IE-1 transcript during the initial stages of reactivation to suppress full lytic gene expression in the reactivating neurons, thus preventing neuronal death.

That LAT must have a function is borne-out by the observations that other alphaherpesviruses have similar patterns of gene expression during latency. LATs have been identified in neurons latently infected with HSV-2 (Mitchell *et al.*, 1990c; Croen *et al.*, 1991; Tenser *et al.*, 1991) which, similar to the HSV-1 LATs, map to the repeat regions of the viral genome. Such patterns of transcription have also been observed for bovine herpesvirus-1 (BHV-1) in rabbits and cattle (Rock *et al.*, 1986, 1987a; Kutish *et al.*, 1990) and pseudorabies virus in swine (Cheung, 1989; Lokensgard *et al.*, 1990; Priola *et al.*, 1990). As with HSV-1, the LAT species of these viruses overlap immediate-early transcription units on the complementary strand (Cheung, 1989; Kutish *et al.*, 1990; Priola *et al.*, 1990). It would seem highly unlikely that the LAT transcription pattern would be conserved in such diverse alphaherpesviruses if LAT were devoid of any important function. Clearly, more extensive studies are required to pinpoint their precise role and function in the pathogenesis of latent infections.

1D.8. IN VITRO LATENCY SYSTEMS

In order to circumvent the problems associated with the studies of latency in sensory ganglia maintained *in vivo*, an *in vitro* latency system would clearly be beneficial. Aside from the problems of proving that *in vitro* cultures are not producing low levels of virus, thereby perpetuating the infection, the major theoretical problem with the development of such systems is that

insufficient information has been available from 'real' *in vivo* systems ie the fundamental characteristics of latent infection that should be attempted to be reproduced in cultured cells. From all of the information gathered so far, studies of cells harbouring episomal DNA and expressing LAT should be the goal.

Over the years, there have been several attempts to study latency in *in vitro* systems, the most extensive reflecting those of Wigdahl *et al.* (1981) whose basic system involved infecting cultured cells (including cultured neurons) at temperatures supraoptimal for virus replication (40°C) and then adding inhibitors of viral replication. Once the 'latent' infection is established the supraoptimal temperature is sufficient to prevent viral replication; when the temperature is lowered to 37°C, virus replication ensues (Wigdahl *et al.*, 1981, 1982, 1983, 1984a). In these systems, transcriptional patterns have not been reported, however, the virus genome appears to persist in some linear form (Wigdahl *et al.*, 1984b).

A variation of the above theme is that employed by Russell & Preston (1986) whereby HSV-2 is converted to a 'latent' state in human foetal lung fibroblasts by increasing the temperature of incubation to 42°C. Viral replication was not usually induced by downshift to 37°C but could be induced by superinfection with *ts* mutants of HSV-1 or with HCMV (Russell & Preston, 1986). It was found that superinfection with *tsK* at 42°C resulted in the rescue of latent virus (presumably by complementation) suggesting that Vmw175 is not necessary for reactivation (Russell *et al.*, 1987b). Interestingly, a Vmw110 mutant (*dl1403*) could not rescue 'latent' virus thereby suggesting that expression of Vmw110 is necessary for reactivation in this system. Further investigation demonstrated that adenovirus recombinants expressing Vmw110 could reactivate latent virus, whereas adenovirus alone could not (Harris *et al.*, 1989). In addition, an HSV-1 mutant possessing a deletion

in the carboxy-terminal region of Vmw110 could reactivate 'latent' HSV-2, while mutant HSV-1 FxE harbouring a deletion in the second exon (containing the putative Vmw110 metal binding domain; see section 1B.4.4) could not, therefore suggesting that this region is important for the reactivation process.

Another model developed by Cook & Brown, (1987), involved the use of supraoptimal temperatures and addition of acycloguanosine to corneal cells infected with HSV-1. Upon downshift and removal of the drug, virus reappeared in cultures where it could not be detected earlier.

In the most recently developed system, latent infection (in which no viral antigens could be detected) was established by incubation of HSV-1 infected primary rat sympathetic neurons in the presence of acycloguanosine and nerve growth factor. When anti-nerve growth factor antibody was added to the cultures, virtually all of the cells began producing viral antigens, and virus reappeared (Wilcox & Johnson, 1987, 1988). ^{Although} the state of the viral genome has not been established, this system has been shown to express LATs (Doerig *et al.*, 1991).

The contribution of these systems to an understanding of latency may or may not be a direct one, however, it is certain that such studies will be informative with respect to the biology of HSV, particularly in relation to an understanding of viral gene expression in nonpermissive cells.

5' [α - ^{32}P] deoxyribonucleoside triphosphate 3000Ci/mmol (10 $\mu\text{Ci}/\mu\text{l}$)

5' [α - ^{35}S] deoxyadenosine triphosphate 1000Ci/mmol (10 $\mu\text{Ci}/\mu\text{l}$)

CHAPTER 2: MATERIALS AND METHODS

2A. MATERIALS

1. Chemicals

Chemicals were supplied by BDH Chemicals UK, Koch-Light Laboratories and Sigma (London) Ltd. Solvents were obtained from James Burroughs UK Ltd and Koch-Light Laboratories.

2. Radiochemicals

All radiochemicals were obtained from NEN Dupont and Amersham International plc.

3. Oligonucleotides

Synthetic restriction enzyme linker oligonucleotides were obtained from New England Biolabs.

4. Enzymes

Restriction endonucleases, DNA modifying enzymes and enzyme buffers were obtained from Bethesda Research Laboratories, New England Biolabs, Nbl Enzymes Ltd. Boehringer Mannheim GmbH and Promega Biotech. DNase, RNase and lysozyme were supplied by Sigma.

5. Cells lines

i) BHK-21 clone 13 cells (Macpherson & Stoker, 1962), a fibroblastic cell line derived from baby hamster kidney cells were used routinely for growth and titration of virus stocks, viral gene expression experiments and short term transfection assays.

ii) C1300 Neuro-2A cells, a murine neuroblastoma cell line obtained from PHLS, Porton Down via Dr. John Quinn (MRC Brain Metabolism Unit, Edinburgh), used for viral gene expression experiments.

6. Tissue culture medium

BHK cells were grown in ETC₁₀, consisting of Glasgow modified Eagle's medium (GMEM; Busby *et al.*, 1964) supplied by Gibco Ltd, supplemented with 10% tryptose phosphate broth, 10% calf serum, 100 units/ml penicillin and 100µg/ml streptomycin.

C1300 cells were grown in Dulbeccos modified Eagle's medium (supplied by Gibco Ltd), supplemented with 10% foetal calf serum, 4mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin.

7. Viruses

All HSV-1 viruses and HSV-1 mutants used or constructed in this study were derived from Glasgow strain 17syn⁺ (Brown *et al.*, 1973).

8. Plasmids The following plasmids were kindly supplied by the acknowledged authors:

- pFJ5 (Rixon & McLauchlan, 1990) was obtained from F.J. Rixon.
- pFJ7 (Rixon & McLauchlan, 1990) contains the promoter of the gene encoding the HSV-2 ribonucleotide large subunit fused to the CAT gene in pFJ5.
- pFJ10 (Rixon & McLauchlan, 1990) contains the promoter of the gene encoding the HSV-2 IE-4/5 gene fused to the CAT gene in pFJ5.
- pUC19 and pUC8, *E.coli* cloning vectors supplied by Bethesda Research Laboratories.
- pT7T3 A dual T7/T3 RNA polymerase expression vector used for sequencing and synthesis of single-stranded radioactive probes supplied by Bethesda Research Laboratories .
- p175 (Everett, 1987b) containing the HSV-1 IE-3 gene under the control of the SV40 early promoter and

enhancer.

- p111 (Everett, 1987b) contains the HSV-1 IE-1 gene under the control of its own promoter.
- pgDCAT (Everett, 1986) contains the promoter of the gene encoding gD (Us6) fused to the CAT gene in the vector pBLW2 (Gaffney *et al.*, 1985).
- pJR3 obtained from C. Preston.
- pGX48 supplied by F.J. Rixon. Contains the HSV-1 *Bam*HI *b* restriction fragment cloned into pAT153.
- pHNF-L (Julien *et al.*, 1987a) contains the coding sequences and 5' flanking sequences of the gene encoding the human neurofilament (large subunit).

9. Bacterial Strains

All plasmids used in this study were propagated in *E. coli* K12 strain DH5 α (F⁻, *endA*1, *hsdR*17, *supE*44, *thi*-1, *recA*, *gyrA*96, *relA*1, *del(argF-lac zya)*U169, ϕ 80d*lacZ* M15 (Hanahan, 1983). Library efficiency *E. coli* DH5 α competent cells were supplied by Bethesda Research Laboratories.

10. Bacterial Culture Medium

- L. Broth 10g/l NaCl, 10g/l bactopectone and 5g/l yeast extract.
- L. Broth agar L. Broth plus 1.5% w/v agar.

Ampicillin, where appropriate, was added to L. Broth or L. Broth agar at 50 μ g/ml.

11. Commonly Used Solutions

- Bradford's reagent: 0.01% coomassie brilliant blue G, 0.003% SDS, 4.75% (v/v) ethanol, 8.5% (v/v) phosphoric acid.
- CLB: 0.5% SDS, 20mM Tris HCl (pH7.5), 2mM EDTA.
- 50x Denhardt's: 1% Polyvinylpyrrolidone, 1% BSA, 1% Ficoll

| | |
|------------------------|---|
| 5x Gel loading buffer: | 5x TBE, 50% glycerol, bromophenol blue, xylene cyanol. |
| Giemsa Stain: | 1.5% suspension of Giemsa in glycerol, heated to 50°C for 2h and diluted with an equal volume of methanol. |
| 2xHeBS: | 260mM NaCl, 9.8mM KCl, 1.6mM Na ₂ HPO ₄ , 11mM D-glucose, 42mM HEPES.(pH7.2) |
| Hybridization mix: | 6x SSC, 5x Denhardt's, 0.1% SDS, 50µg/ml denatured calf thymus DNA. |
| 10x NT buffer: | 0.5M Tris HCl (pH7.5), 100mM MgCl ₂ , 100mM DTT, 500µg/ml BSA. |
| PBSA: | 170mM NaCl, 3.4mM KCl, 10mM Na ₂ HPO ₄ , 2mM KH ₂ PO ₄ (pH7.2). |
| PBS: | PBSA plus CaCl ₂ .H ₂ O and MgCl ₂ .6H ₂ O both at 1g/l. |
| 20xSSC: | 174 g/l NaCl, 88.2 g/l Trissodium citrate. |
| TBE: | 125mM Tris, 40mM Boric acid, 2.7mM EDTA, not pH'd. |
| TE: | 10mM Tris HCl 1mM EDTA, pH8.0. |
| TEN: | 150mM NaCl, 40mM Tris HCl, 1mM EDTA, pH7.5. |
| Trypsin: | 0.25% w/v trypsin dissolved in tris-saline. |
| Versene: | 0.6mM EDTA dissolved in PBSA containing 0.0002% w/v phenol red. |

12. Animals

Balb/c mice, obtained from Bantin & Kingman Ltd (England), were used for all animal latency experiments.

2B. METHODS

2B.1. CONSTRUCTION AND PREPARATION OF PLASMIDS

1.1. Restriction Enzyme Digests

Diagnostic restriction enzyme digests were generally carried out in 20 μ l volumes of the appropriate buffer as specified by the manufacturers. The number of units of enzyme added was dependent on the activity of the enzyme and the amount of DNA being digested. Reaction mixtures were generally incubated at 37°C for 1h. Preparative restriction enzyme digestions used increasing amounts of DNA and restriction enzyme under similar conditions in 50 μ l volumes.

For the production of ^{singly cut} linear molecules, DNA (2 μ g) was digested with 2 units of enzyme in the presence of 10 to 500 μ g/ml of ethidium bromide to identify the optimum conditions for production of a maximum of singly cut molecules.

1.2. Separation of DNA Fragments by Non-Denaturing Gel Electrophoresis

a) Agarose gels: 200ml horizontal slab gels (260mmx160mmx5mm) containing 0.8% to 1% (w/v) agarose were electrophoresed submerged in 1x TBE plus 0.5 μ g/ml ethidium bromide at up to 12V/cm. Samples were loaded in 0.2 vol loading buffer. Gels were run until the bromophenol blue ran almost the whole length of the gel. DNA was examined and photographed with short-wave uv transillumination (long wave was used to reduced DNA damage when preparative gels were being analysed).

50ml 'mini' agarose gels (100mmx70mmx7mm) were also used, typically for analysis of mini-prep DNA. Typically, these gels were electrophoresed in TBE at 100V for 1hr.

b) Polyacrylamide gels: In order to accurately resolve DNA fragments of 40-400bp in length, 5% polyacrylamide

(acrylamide:bis-acrylamide, 19:1) gels were run in 1x TBE. Gels were prepared in 1.5mm thick vertical glass plate sandwiches, and cross-linked with 0.001 vol TEMED, catalysed with 0.01% APS. Samples were loaded as above and electrophoresed at a maximum voltage of 16V/cm, and DNA visualised by autoradiography or EtBr staining.

1.3. Purification of DNA Fragments from Gels

a) Agarose gels: The agarose slice containing the required DNA fragment was removed from the gel. The gel slice was placed in dialysis tubing and the DNA isolated from the agarose by electroelution in 1xEEB at 50V for 1hr. The EEB containing the DNA was removed from the dialysis tubing, extracted with phenol/chloroform and precipitated with 2 volumes of ethanol at -20°C for 1-2hr.

b) Polyacrylamide gels: The polyacrylamide slice containing the appropriate fragment was cut into small pieces, and the DNA eluted into TE by shaking overnight at 37°C. The TE was then removed, extracted with phenol/chloroform and precipitated with 2 volumes of ethanol at -20°C for 1-2hrs.

1.4. DNA Ligation

DNA ligations were performed in 20µl 1x ligase buffer, with 0.5mM ATP, 50-500ng DNA and 1 unit of T4 DNA ligase. To prevent self-annealing of the vector, it was usually treated with calf intestinal phosphatase during the preceding restriction enzyme digestion.

Staggered cut termini of DNA fragments were converted to blunt-ends by treatment with T4 DNA polymerase. Reactions contained 0.5 to 1.0µg DNA, 33mM Tris HCl (pH7.8), 66mM potassium acetate, 10mM magnesium acetate, 100µg/ml BSA, 200µM dCTP, dGTP, dATP, dTTP plus 4 units of T4 DNA polymerase and were incubated at 15°C for 3hrs.

Phosphorylated oligonucleotide linkers were inserted into plasmids by ligation with linear molecules

using the same procedure as for vector/fragment ligations. A 50-fold molar excess of linker over plasmid DNA was used.

1.5. Transformation of competent *E. coli*

1 μ g of ligation mix was added to 20 μ l of competent DH5 α cells and incubated on ice for 30 mins. The mixture was then heat-shocked at 42°C for 45 secs and added to 80 μ l of SOC medium (2% bactopeptone; 0.5% yeast extract; 10mM NaCl; 2.5mM KCl; 10mM MgCl₂; 10mM MgSO₄; 20mM glucose). Cultures were then agitated at 37°C for 1hr, and subsequently spread on L. Broth agar plates containing ampicillin. If blue/white selection was used as in the case of pUC cloning vectors, 10 μ l of 150mg/ml X-gal diluted in 100 μ l ethanol was spread on the amp plates and allowed to dry prior to spreading of cultures. The plates were then incubated at 37°C overnight.

1.6. Mini-Prep Analysis of Transformed *E. coli* Colonies

Mini-prep DNA was prepared by the alkaline lysis procedure. Colonies were picked from agar plates into 5ml of L. Broth containing ampicillin and shaken at 37°C overnight. The following day, *E. coli* cells from 1ml of the overnight culture were pelleted at 5,000rpm for 1min in a benchtop microfuge. The pellet was resuspended in 100 μ l of solution 1 (50mM glucose, 10mM EDTA, 25mM Tris pH8.0) containing 1mg/ml lysozyme and incubated at RT for 5min. 200 μ l of solution 2 (0.2M NaOH, 1% w/v SDS) was then added and the mixture vortexed. 150 μ l of solution 3 (3.0M KAc, 11.5% v/v glacial acetic acid) was then added, and following vortexing, was incubated on ice for 10 mins. This mixture was centrifuged at 13,000rpm for 5min and the pellets discarded. The supernatant was precipitated with an equal volume of isopropanol at -20°C for 20mins. After centrifugation at 13,000rpm for 5min in a benchtop microfuge, the pellets were washed in ethanol, dried and resuspended in 50 μ l of H₂O.

Mini-prep DNA was stored at -20°C and $5\mu\text{l}$ used for restriction enzyme analysis. $0.2\mu\text{l}$ of 1mg/ml RNase was added to digestions of mini-prep DNA. Samples were generally assayed for the insertion of a fragment by screening for an increase in size compared with the vector DNA. Further analysis was carried out by restriction enzyme digestion of positive samples.

For long term storage, glycerol stocks of positive cultures were made by pelleting 1ml of culture by centrifugation at $6,000\text{rpm}$ in a benchtop microfuge and resuspending in $500\mu\text{l}$ bactopectone plus $500\mu\text{l}$ 80% glycerol. Glycerol stocks were stored at -70°C .

1.7. Large Scale Preparation of Plasmid DNA

Transformed bacteria from the appropriate glycerol stock were picked and inoculated into 10ml of L. Broth plus ampicillin and incubated at 37°C overnight. 0.5ml of this stock was used to inoculate 300ml of L. Broth plus ampicillin and the culture shaken shaken at 37°C for 8h . Chloramphenicol was then added to a final concentration of $25\mu\text{g/ml}$ and incubation continued overnight. The culture was pelleted by centrifugation at $7,000\text{rpm}$ for 10min in a Sorval GS3 rotor. After decanting the supernatant, the pellet was resuspended in 7.5ml of solution 1 containing 1mg/ml lysozyme. After leaving at RT for 5min , 14ml of solution 2 was added, and following vortexing, was left in ice for 10min . 15ml of solution 3 was subsequently added, and following further vortexing, was left on ice for a further 15min . This was then centrifuged at $17,000\text{rpm}$ for 30min in a Sorval SS34 rotor to pellet out the cell debris and cellular DNA. The supernatant containing the plasmid DNA was precipitated with an equal volume of isopropanol at -20°C . DNA was pelleted by centrifugation at $3,000\text{rpm}$ for 15min in a Sorval RT6000B benchtop centrifuge, washed in ethanol, dried and resuspended in a total volume of 1ml of H_2O .

This solution was prepared for caesium banding by dissolving 1.15mg of CsCl and adding 50 μ l of 10mg/ml EtBr per ml of DNA. This was transferred to a Beckman TLV-100 ultracentrifuge tube and topped up with stock solution containing similar amounts of CsCl and EtBr. The ultracentrifuge tube was then heat-sealed and centrifuged for 4.5h at 100,000rpm or 16h at 80,000rpm at 20°C in a Beckman TL100 benchtop ultracentrifuge. DNA was visualised by daylight or long wave uv transilluminator and the lower supercoiled plasmid DNA band recovered with a large bore needle and syringe. The DNA was extracted three times with isopropanol/saturated CsCl solution to remove EtBr, and its volume increased by a factor of three with H₂O, and precipitated with ethanol. Once resuspended in TE, the DNA was treated with 50 μ g RNAase (1h/65°C) before extraction with phenol/chloroform and chloroform and precipitation with ethanol. The DNA was lyophilized, resuspended in H₂O and its concentration determined by spectrophotometry (OD₂₆₀ 1.0=50 μ g DNA/ml).

2B.2. FUNCTIONAL ANALYSIS OF PLASMIDS

2.1. Transfection of Plasmid DNA into Cells

Plasmid DNA was transfected into BHK cells using a modified version of the calcium phosphate technique used by Shen *et al.* (1982). 3 μ g of plasmid was typically used for each transfection. 3 μ g of plasmid DNA was made up to 57 μ l in deionised H₂O, and 66 μ l of 2xHeBS added. 9 μ l of 2M CaCl₂ was squirted in, and the samples immediately vortexed. The tubes were allowed to stand at room temperature for 10mins. The medium was then removed from 80% confluent cell monolayers grown in 35mm petri dishes, and overlaid with the calcium phosphate precipitate and incubated at 37°C for 45mins with intermittent rocking.

2ml of medium (consisting of multiples of: 18.75 conditioned medium, 20ml ETC₁₀, 2ml 2xHeBS, 0.25ml CaCl₂;

the latter added just before overlaying on cells) was added to each monolayer and the incubation continued for a further 3-4h at 37°. The medium was then removed from the cells and 25% v/v dimethyl sulphoxide in 1xHeBS applied for 4 min at room temperature (Stow & Wilkie, 1976). The DMSO was removed and the cells washed twice with ETC₁₀; a further 2ml of ETC₁₀ was added and the monolayers incubated at 38.5°C. Infection with virus, when required, was performed after 1h. Incubation was then continued overnight at 38.5°C.

2.2. Preparation of CAT Extracts

Extracts were prepared by washing the cells in PBSA then scraping into 2ml of TEN and transferring to 15ml falcon tubes. The cells were pelleted at 2000rpm/4°C/1min in a Sorval RT6000B centrifuge and resuspended in 75µl 0.25M Tris pH7.8 and lysed by sonication. The sonicates were then transferred to 1.5ml eppendorff tubes and centrifuged at 13,000rpm for 2min in a benchtop microfuge to remove cell debris. The cell extract supernatants were stored at -20°C.

2.3. CAT Assays

Cell sonicates were assayed for chloramphenicol acetyltransferase activity essentially as described by Seed & Sheen, (1988). Assay mixtures containing 1µl 25mM *N*-butyryl-CoA, 0.5µl stock [¹⁴C]CAP (45µCi/mM), 5µl 250mM Tris HCl pH7.8 and 18.5µl H₂O together with 20µl neat or diluted sonicate. After incubation for 30 or 60min at 37°C, the products were extracted with 150µl TMPD/Xylenes (2:1 mixture respectively). The percentage conversion to product was determined by scintillation counting of 140µl of the TMPD/Xylenes extract in 3ml ecoscint as a fraction of the total counts in the assay (the total number of counts in a parallel control reaction). The protein concentration of the extract was determined by the method of Bradford

(1976). CAT activities were expressed as the percentage [^{14}C] chloramphenicol converted to the butyrylated form per μg protein per h.

2.4. Determination of Protein Concentration in Cell Extracts

The protein concentration of CAT extracts was determined by the method of Bradford (1976). 2.5–5 μl of extract was made up to 100 μl in 0.05M Tris.HCl pH7.8 and 1ml of Bradford's reagent added and mixed. After 15min incubation, the OD₅₉₅ was measured in disposable plastic cuvettes by spectrophotometry. The protein concentration per μl of extract was determined from a standard curve constructed using serial dilutions of BSA from 5 to 80 $\mu\text{g}/\text{ml}$.

2.5. In Vitro Transcription of Plasmids

Plasmids were digested with a restriction enzyme to produce linear molecules that were cleaved 3' to the coding sequence. *In vitro* transcription was performed using the T7T3 Riboprobe system (Bethesda Research Laboratories) following the manufacturers protocol. 1 μl of linear DNA was mixed at RT with 5 μl SP6 buffer (200mM Tris.HCl (pH7.5), 30mM MgCl₂, 10mM spermidine), 0.01% BSA, 10mM DTT, 0.4mM ATP, UTP, CTP, GTP, 0.5mM G(5')ppp(3'), 40 units RNAasin (Promega Biotech) and 10 units T7 RNA polymerase in a total volume of 25 μl . The reaction mixtures were incubated at RT for 1h.

2.6. DNA Sequencing

DNA fragments cloned into pT7T3 were sequenced using the Pharmacia T7 sequencingTM kit consisting of dideoxy sequencing reactions using T7 DNA polymerase. This was carried out according to manufacturers instructions.

2.7. Denaturing Polyacrylamide Gels

An Acrylamide solution was prepared which contained 9ml of 40% acrylamide/ N, N'-methylene-bisacrylamide (20:1), 28.8g Urea, 3ml 10xTBE, 25ml H₂O, 100µl 25% APS and 100µl TEMED. This solution was poured into a mould (230mmx450mmx0.35mm), and after polymerisation was prerun at 40W for 30min prior to loading of samples. Electrophoresis was performed in 0.5x TBE at 40W for approximately 2h.

2B.3. CONSTRUCTION AND PREPARATION OF VIRUSES

3.1. Tissue culture

BHK cells were routinely passaged in 850cm² roller bottles seeded with approximately 2x10⁷ cells at 37°C in 100ml ETC₁₀ and an atmosphere of 5% CO₂ in air. Confluent monolayers were harvested in 20ml ETC₁₀ after two versene washes and brief trypsinization with trypsin:versene (1:4). Cells were resuspended by pipetting and remained viable for at least 5 days stored at 4°C.

C1300 cells were routinely passaged in 175cm² (Nunclon) flasks in DMEM and harvested in 10ml. C1300 cells did not remain viable at 4°C and for long term storage, harvested cells were pelleted and resuspended in medium plus 5% glycerol and 15% foetal calf serum, aliquoted and frozen slowly to -140°C. Recovery was by rapid thawing and resuspension in growth medium.

3.2. Preparation of Stocks of Infectious Virus

80% confluent monolayers of BHK cells were seeded with 4x10⁴ pfu of virus per roller bottle in 40ml ETC₁₀ medium (a moi of 0.002pfu/cell) and incubated for 4-5 days at 31°C until the cells exhibited obvious cpe (31°C rather than 37°C was found to be better for growth for high titre viral stocks). The cells were shaken into medium and pelleted at 1,000rpm/ 5min/ 4°C. Cell associated virus (CAV) was prepared by sonicating the pellet in 2ml ETC₁₀.

Cell released virus (CRV) was pelleted from the supernatant at 9,000rpm/ 2hours/ 4°C and gently resuspended in 2ml ETC₁₀ and sonicated. Sterility checks were performed by streaking virus preparations on blood agar plates and incubating at 37°C. Virus stocks were stored at -70°C.

3.3 Purification of Virus Particles

Virions were prepared from monolayers of BHK cells grown in roller bottles. Once the cells exhibited obvious cpe, cells were shaken into the medium, and cell debris removed by low speed centrifugation (5000 rpm for 10 min at 4°C), and the virus particles in the clarified medium were pelleted by centrifugation (12,000 rpm for 2h at 4°C). The pellet was then gently resuspended in 1 ml of Eagle's A + B (without phenol red and calf serum) and layered onto a 35 ml preformed gradient of 5 to 15% Ficoll 400 (Sigma) suspended in this medium. After centrifugation in a swing-out rotor (12,000 rpm for 2h at 4°C in an AH627 cellulose nitrate tube), the lower particle band was withdrawn by side punctures. These virions were pelleted by centrifugation (21,000 rpm for 2h at 4°C in an AH627 tube), gently resuspended in 200µl of modified medium and either used immediately or stored at -70°C.

3.4. Preparation of virion DNA

DNA was extracted from purified virions by treatment with SDS and proteinase K (0.5% and 50 mg/ml respectively) for 1h at 37°C, followed by phenol/chloroform extraction (3 times) and precipitation by addition of 2 volumes of ethanol. For the purposes of the HSV vector, purified DNA was digested to completion with *Xba*I, divided into aliquots, and stored at -20°C.

3.5. Titration of virus stocks

BHK cells were seeded at 2×10^6 cells per 50mm plate in 5ml medium. The following day, cells were infected with

serial 10-fold virus dilutions in 0.1ml of PBS/ 10% newborn calf serum. After 1h adsorption time, plates were overlaid with 4ml 0.6% Noble agar medium (100ml 1.3x Eagle's A, 20ml Eagle's B without phenol red, 30ml 3.2% Noble agar, 5ml serum) to prevent secondary plaque formation, and incubating for 2-3 days at 37°C prior to fixing with Cidex and staining with Giemsa stain. The plates were washed after 15min and plaques counted using a plate microscope.

3.6. Construction and Screening of Recombinant Virus

Plasmid DNAs cleaved with *Xba*I were electrophoresed on agarose gels from which the appropriate fragments were isolated and purified. These fragments were ligated with *Xba*I-digested virus DNA prior to transfection onto monolayers of BHK cells. Transfections were performed using the calcium phosphate precipitation/DMSO method described by Stow & Wilkie (1976). This was essentially as described in section 2B.2.1, except that 2.0µg of calf thymus DNA was used as carrier and precipitates were gently mixed upon addition of CaCl₂ and not vortexed. Following transfection, the cells were either overlaid with 0.6% Noble agar medium (100ml 1.3x Eagle's A, 20ml Eagle's B without phenol red, 30ml 3.2% Noble agar, 5ml serum) or were overlaid with ETC₁₀ for subsequent harvesting and titrating of progeny virus. Titrations were performed under agar medium. Two days after transfection or infection, a further 2ml of agar medium containing 0.75mg/ml X-gal was added to each plate. After incubation overnight, plaques expressing β-galactosidase were picked. All viruses were purified to homogeneity typically through three to four rounds of plaque purification.

2B.4. ANALYSIS OF VIRAL DNA

4.1. Preparation of Total Infected Cell DNA

10mm linbro wells were seeded with about 4×10^5 BHK cells and infected the following day with half of a picked plaque or a moi of 1 pfu/cell. After about 2 days when cpe became apparent, the supernatant was removed and stored as CRV stock. 0.2ml of cell lysis buffer (CLB) containing 0.25mg/ml proteinase K was added to the cell monolayer and incubated at 37°C for 3-5h. The glutinous extract was transferred to 1.5ml Eppendorff tubes and 15 μ l 5M NaCl added. DNA was extracted with phenol/chloroform followed by precipitation with 2.5 volumes of ethanol. The pellet was then washed with 70% ethanol, dried, and finally resuspended in 50 μ l H₂O.

4.2. Internal Labelling of Probes by Nick Translation

DNA was labelled with ³²P as described by Rigby *et al.* (1977). 1.0 μ g of plasmid DNA was incubated in a reaction mix containing 40mM dATP, 40mM dTTP, 2.5 μ l of 10x NTB (5M Tris HCl (pH7.5), 1M MgCl₂, 1M DTT and 5mg/ml BSA) and 2×10^{-4} μ g DNAase at RT for 3min. The mix was placed on ice and diluted to 25 μ l by the addition of 30 μ Ci of both α -[³²P]-dCTP and α -[³²P]-dGTP, 3units of *E.coli* DNA polymerase I plus distilled H₂O. The reaction was incubated at 15°C for 1h. ³²P labelled DNA was then separated from unincorporated triphosphates by running through a 10ml Sephadex (medium) G50 column in a 10ml pipette plugged with siliconized glass wool. Columns were loaded with the sample with bromophenol blue dye and the fastest eluting fractions (detected by Geiger counter) taken as purified DNA.

4.3. Transfer of DNA to Nitrocellulose

The method used was essentially that of Southern, (1975). The agarose gel to be blotted was shaken for 45min in Gel Soak I (200mM NaOH, 600mM NaCl), then for a further 45min in Gel Soak II (1M Tris HCl (pH8.0), 0.6M NaCl) at room temperature. The gel was transferred to two sheets of Whatman 3MM filter paper, supported by a glass plate. the

edges of the filter paper were dipped into a tray containing 6x SSC. A sheet of nitrocellulose, the same dimensions as the gel was placed on top, covered by three sheets of 3MM paper and a stack of paper towels, also cut to size. The towels were weighted and the blot left overnight to allow the DNA to transfer to the nitrocellulose. The following day, the nitrocellulose was removed and allowed to dry in air and baked at 80°C for 2h.

4.4. DNA/DNA Hybridisation

Hybridisations were carried out under conditions of high stringency by the method of Southern *et al.* (1975). The nitrocellulose, bearing separated DNA fragments, was prehybridised in 100ml 6xSSC, 5x Denhardt's and 20µg/ml denatured calf thymus DNA at 65°C for 2h in a sealed polythene bag. The probe was meanwhile denatured by incubation in 0.2M NaOH for 10min at RT, followed by neutralisation by the addition of 0.2M HCl.

The prehybridisation mixture was removed and replaced with the denatured probe in a mix of same final composition as the pre-hybridisation solution. The nitrocellulose filter was shaken overnight at 65°C, then washed in 10x denhardt's, 4x SSC for 30min at RT, followed by three washes in 0.3x SSC, 0.1% SDS at 65°C. The filter was then air dried and autoradiographed.

2B.5. ANIMAL STUDIES

5.1. Footpad Inoculation

Female weanling (3-4 weeks) Balb/c mice were injected with the appropriate dose of virus (in 25µl) into the right rear footpad using a 26G (3/8) needle. The remaining virus suspension was titrated on BHK cells after each inoculation. Mice were examined daily for the first two weeks after inoculation for signs of paralysis; any showing any ill-effect were immediately put down.

5.2. Explantation and Cultivation of Dorsal Root Ganglia from Latently Infected Mice

Latently infected mice ^(4 wks pi) were sacrificed by chloroform inhalation and fixed on a dissection board in a dorsal ventral position. The back of the mouse was carefully washed in 70% alcohol. The skin was dissected away from the body, and the spinal column cleared of fat and supporting muscle. A transverse cut was made through the vertebral column at the level of the neck, and the dorsal lamina of the vertebral column separated and removed. The spinal cord was then carefully removed and the dorsal root ganglia identified under a dissecting microscope.

5.3. DRG Organ Culture

Nine DRG's were explanted from the right hand side comprising the last two thoracic ganglia, all six lumbar ganglia, and the first sacral ganglia. Lumbar ganglia from the left hand side were also explanted as internal controls. The explanted DRG were ^{leaved apart} washed separately in PBS/10% foetal calf serum, then placed into separate wells of a microtitre plate containing 150µl EFC₅₀ per well. The microtitre plates were incubated at 37°C in a humidified incubator under an atmosphere of 5% in air. The explanted ganglia were screened daily for the presence of released HSV by plating 0.1ml of culture supernatant onto BHK cells and observed for cpe.

5.3. In Situ Hybridisation

a) Pretreatment

Ganglia were fixed in freshly made 4% paraformaldehyde in 0.1M phosphate buffer for 1h at 4°C. The fixative was prepared by dissolving 1g of paraformaldehyde in 10ml dH₂O with 2 drops of 1M NaOH added and heating to 60°C, then cooled and 12.5ml 0.2M phosphate buffer (9.5ml 0.4M NaH₂PO₄ + 40.5ml Na₂HPO₄, made up to

100ml and pH adjusted to 7.4) added. The paraformaldehyde was made up to 25ml with dH₂O and pH adjusted to 7.3. The ganglia were then washed in 70% ethanol and embedded in blocks of 1% noble agar. The agar blocks were then further paraffin embedded.

The paraffin embedded sections were dewaxed with Xylenes, and rehydrated in absolute alcohol, 95% alcohol, saline, and PBS for 5mins each. These were post-fixed in 4% paraformaldehyde (5min), washed twice in PBS (2mins each) and digested in 20µg/ml proteinase K (in 10mM Tris pH7.4, 2mM CaCl₂) for 7½ mins. Following washing in PBS, the sections were again post-fixed in 4% paraformaldehyde (5min). The sections were then acetylated by treatment 0.1M triethanolamine with acetic anhydride being added to 0.25% (with stirring for 5min) and then a further aliquot of acetic anhydride being added to 0.5% (stirred again for 5min). The sections were then dehydrated by washing in PBS (2min), saline (2min), 95% alcohol (2min), and absolute alcohol (5min). The slides were then air dried and stored at RT until ready for hybridization.

b) Hybridization

The radiolabelled probe (specific activity greater than 0.2×10^8 ; 2.5×10^5 precipitable counts per section) was made up in hybridization solution (2xSSC, 50% Formamide, 10mM Tris pH7.4, 1mM EDTA, 1x Denhardt's, 10% Dextran sulphate). This was heated to 100°C for 30sec, quenched on ice, and DTT added to 10mM final concentration. 5µl of this mix was added to each section and a coverslip placed on top. The slides were sealed in a box with a tissue soaked in 50% formamide, and incubated at 50°C for 18h.

c) Post-Hybridisation Washes

Following hybridization, the slides were washed in wash solution 1 (5xSSC, 10mM DTT at 50°C) for 10min, then

wash solution 2 (50% Formamide, 2xSSC, 10mM DTT at 65°C) for 20min, then wash solution 3 (0.5M NaCl, 10mM Tris pH7.5, 5mM EDTA at 37°C) for 5min, then again in fresh solution 3 for a further 5min. The sections were then treated in with RNAase (20µg/ml in solution 3) at 37°C for 30min, then further washed in solution 3 (37°C, 10min), solution 2 (65°C, 20min), 2xSSC (10min at RT), 0.1xSSC (10min, RT) then dehydrated in 30%, 60%, 80%, 90% and 100% alcohol (each containing 0.3M NH₄Ac) for 2min each, and finally absolute alcohol (2min). The slides were air dried overnight at RT, and stored for up to 2 days in a dust free container.

d) Dipping into Photographic Emulsion

This procedure was carried out in a dark room with all lights (including safety lights) out. K5 emulsion (Amersham) (enough for 7.5 ml) was melted in a water bath pre-warmed to 45°C and gently mixed with an equal volume of glycerol/H₂O (pre-warmed to 45°C). This mixture was pipetted slowly (to avoid bubbles) into an Amersham dipper. The slides were dipped slowly into the emulsion, and removed slowly in one motion. The slides were left to dry for 3h, then placed in a light proof box with silica granules, and put at 4°C until development.

e) Development of Emulsion

Slides were developed in D19 developer (Ilford) for 4min, washed in H₂O for 30sec, then fixed in hypam (Ilford) (1 in 5 dilution with H₂O) for 4min. All these steps were carried out at 16°C. The slides were washed in running water, wiped, and examined under a microscope for grains.

f) Counterstaining Slides

The slides were washed in haematoxylin (5min), rinsed in tap water, then hard water (2-3min until they turned blue). They were then washed in Eosin (3-5min),

rinsed briefly in H₂O and dehydrated in 95% and 100% alcohol (2min each) then histoclear (3min). The sections were then mounted in DPX by touching the slide onto a DPX coated coverslip.

5.5 HISTOCHEMICAL STAINING OF GANGLIA

Spinal ganglia were removed from mice and fixed for 1h in paraformaldehyde in 0.1% phosphate buffer. Ganglia were then rinsed in PBS and incubated for 4-6h at 31°C in a solution containing 1.0mg/ml X-gal, 0.1% sodium deoxycholate, 0.02% Nonidet P-40, 5mM potassium ferricyanide, 5mM ferrocyanide and 2mM magnesium chloride in PBS. Incubation for any longer than 6h led to a high level of background staining. Controls without X-gal were run since blue/green colour can be produced by Fe³⁺ in cells reacting with ferrocyanide (ferricyanide catalyses oxidation of liberated indolyl to insoluble indigo dye; ferrocyanide prevents further oxidation to a colourless dehydroindigo compound). The tissues were thoroughly rinsed in PBS and examined as whole mounts. They were then wax-embedded and sectioned at 6µm, counterstained with eosin and evaluated by light microscopy.

CHAPTER 3: RESULTS

3A. OBJECTIVES

The aim of the work presented in this thesis was to investigate the *cis*-acting control sequences of HSV and non-HSV promoters under conditions which are permissive and non-permissive for viral replication. The ultimate objective was to introduce promoter/reporter gene fusions into the genome of a virus vector developed by Rixon & McLauchlan (1990), and examine their expression in the context of virus genome.

When HSV-1 establishes latency in peripheral nervous system ganglionic neurons, detectable RNA transcription is confined to one locus of the virus genome. As described in section 1D.7.2, this results in a family of co-linear RNAs termed the latency associated transcripts (LATs; see section 1D.7.2). Being the only gene transcribed from the latent HSV-1 genome suggests that its controlling elements must differ considerably from those controlling lytic cycle gene expression. A substantial body of work presented in this thesis is therefore devoted to the mapping and characterisation of the LAT promoter. This was carried out by sequence analysis, coupled with the cloning of various upstream sequences of the LAT gene and examination of their ability to drive expression of an adjoining reporter gene in transient transfection assays, and when introduced into the viral vector, during infection of tissue culture cells. During the course of this study, the discovery by other groups of an 8.3 kb LAT demonstrated that the LAT gene transcription pattern is more extensive than previously thought. For the purposes of this study, though, reference will only be made to the 2 kb LATs, since the strategy for mapping the LAT promoter was based upon the mapping of these original transcripts. The relevance of the more extensive pattern of LAT transcription will be discussed later.

Since HSV establishes latency in neurons, an

intriguing question to ask is how an endogenous neuronal promoter would behave in the context of the latent HSV genome. To this end, the upstream sequences of the human neurofilament light-chain (HNF-L) gene were acquired and characterised for promoter activity in transfection assays. When introduced into the viral vector genome, NF-L promoter/reporter gene constructs were further characterised during lytic infection of tissue culture cells and in animal latency systems.

3B. CHARACTERISATION OF THE LAT PROMOTER

3B.1. Sequence Numbering Convention

Throughout the following sections dealing with HSV-1 LAT promoter characterisation, the sequence numbering convention used is such that the mapped 5' end of the 2kb LAT, ie the distal G residue in the sequence 5'-.AGGT.-3', at position 119461 of the published HSV-1 sequence (Perry & McGeoch, 1988) is referred to as position zero.

3B.2. Sequence Analysis of the Putative LAT Promoter Region

Examination of the HSV-1 strain 17+ sequence immediately upstream of the 5' end of the 2 kb LAT does not reveal any reasonable consensus RNA polymerase II promoter elements. Indeed, the first recognisable pol II elements occur much further upstream than expected for a normal lytic cycle gene. The salient features of this potential promoter are a TATA box at position -689 to -681 (...CCTTTATAAAAGC...), a CAAT box at position -818 to -815 (...ATCAATCC...) and two putative Sp1-binding sites at positions -864 to -859 (...CCCCGCCCG...) and -887 to -882 (...CCCCGCCCC...). A further putative Sp1 site is located downstream of the TATA box at position -590 to -585 (...CGGGCGGCC...). The combination of these sequence motifs, together with their positions relative to each other, earmark this region as an excellent candidate for an

RNA polymerase II promoter. Wagner *et al.* (1988) when analysing the DNA sequence immediately surrounding the LAT 5' terminus of HSV-1 strain KOS(M) identified other sequences which contained some minimal features of a pol II promoter. These included a possible TATA box homologue at position -24 to -16 (...GTTTCAACAAAGA...) and potential CAAT box homologies at positions -50 to -47 (...CCCACTCG...) and -86 to -83 (...CCCAGTAC...). Another potential promoter within the LAT region is located far downstream of the LAT 5' terminus and consists of a TATA homologue at position 827 to 830 (...CCTATAGT...) with accompanying putative Sp1 binding sites at positions 733 to 738 (...CTCCGCCCCA...), 794 to 799 (...TCCCGCCCCG...), 848 to 853 (...CCCGCCCTT...) and 883 to 888 (...CCGGGCGGGC...). Although it is possible that these elements could form a pol II promoter, their positions relative to the 5' end of LAT suggest that it is highly unlikely that they could have any part to play in transcriptional regulation of the LAT gene.

3B.3. Comparison of HSV-1 and HSV-2 Upstream LAT Sequences

Since LATs are now known to be transcribed from latent HSV-2 genomes (Mitchell *et al.*, 1990; Croen *et al.*, 1991; Tenser *et al.*, 1991), the sequences responsible for transcriptional regulation of the HSV-1 and HSV-2 LAT genes are presumably conserved to some degree. A direct sequence comparison of the corresponding LAT regions of HSV-1 and HSV-2 should therefore reveal conserved sequences which could be considered as candidates for the LAT promoter region.

A sequence comparison was therefore carried out using sequencing data from HSV-1 strain 17+ (Perry & McGeoch, 1988) and unpublished sequencing data of the R_L region of HSV-2 strain HG52 (kindly supplied by Dr. Duncan McGeoch). Sequences were compared with the Vax GCG program Compare and plotted on a panel illustrated in Figure 6

using the GCG program Dotplot. The results of this sequence comparison resemble those of an analysis carried out by Dr. Duncan McGeoch. HSV-1 is on the horizontal axis which consists of 1600 bp corresponding to bases 118000 to 119600 of the published HSV-1 sequence. A schematic representation of the major features of this region is shown above the horizontal axis. The mapped 5' terminus of LAT is indicated at position 0 and corresponds to HSV-1 residue 119461. The corresponding region of HSV-2 is on the vertical axis.

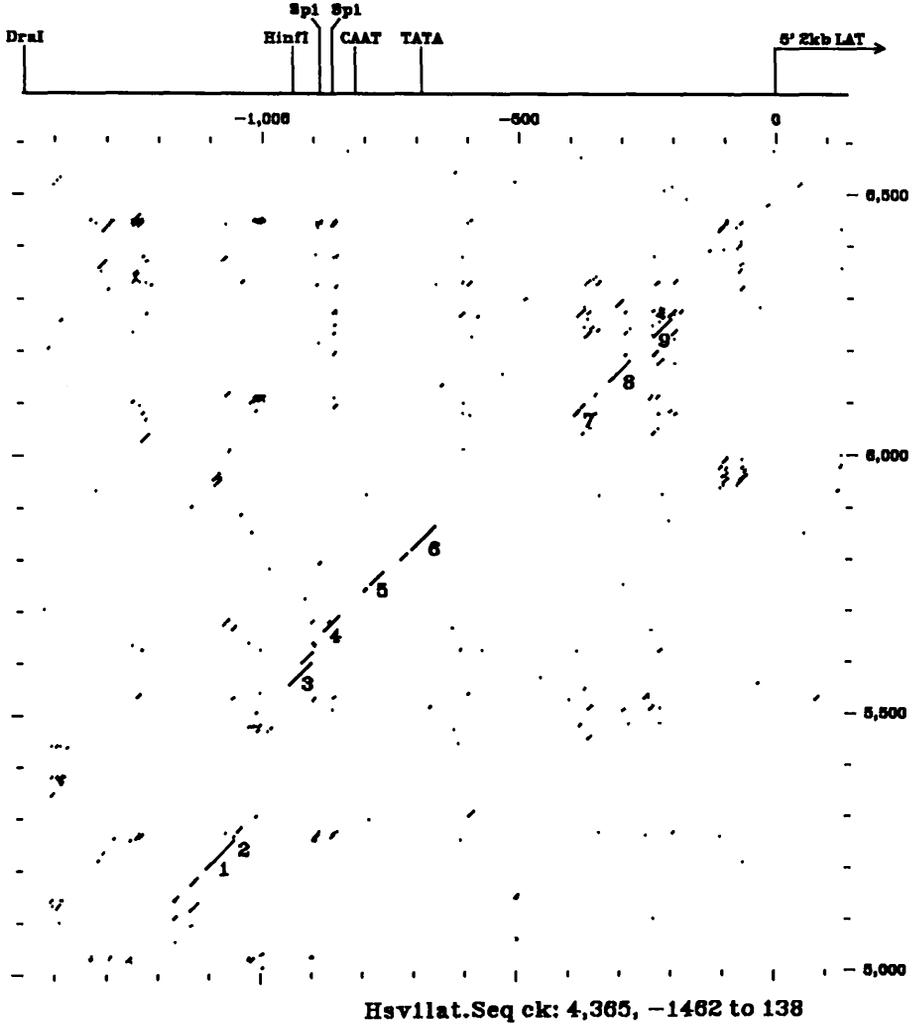
This comparison indicates that large regions of R_L around the 2 kb LAT specifying sequences are widely diverged; the only detectable related sequences are numbered on Figure 6. Interestingly, the 2 kb LAT specifying sequences fail to show any degree of significant conservation, indeed, the only conserved region downstream from the 5' terminus of LAT is that corresponding to the IE-gene 1 protein coding region (data not shown). All of the conserved loci are upstream from the mapped 5' end of the 2 kb LAT and include the region bearing most resemblance to a pol II promoter. In all, there are 9 short conserved loci designated 1 through to 9 as indicated on Figure 6. An alignment of the actual sequences corresponding to these loci is shown on Figure 7; HSV-1 sequence is the upper, HSV-2 is the middle and the consensus is shown on the bottom line. Figure 7 is an extended version (by 180 bp) of a sequence comparison generated by Dr. Duncan McGeoch using the GCG program Gap.

Both Figures 6 and 7 demonstrate a complete lack of homology in the sequences immediately surrounding the HSV-1 LAT 5' terminus. This made it unlikely that the putative elements identified by Wagner *et al.* (1988) formed the *bona fide* LAT promoter. The upstream consensus motifs are, however, well conserved. The predicted TATA box at -689 to -681 occurs within consensus locus 6 and is perfectly conserved in the HSV-2 sequence. The predicted HSV-1 CAAT box located between conserved loci 4 and 5 has no HSV-2

Figure 6. Sequence comparison of R_L regions of HSV-1 17+ and HSV-2 HG52. Sequences were compared with the GCG program Compare, and plotted on the panel illustrated using the GCG program Dotplot. The HSV-1 sequence on the horizontal axis consists of 1600 bp corresponding to 118000 to 119600 of the published HSV-1 sequence (Perry & McGeoch, 1988). A schematic representation of the major features of this region including positions of various putative promoter elements and restriction enzyme sites are shown above the horizontal axis. The mapped 5' terminus of the 2 kb LAT is indicated at position 0, and corresponds to HSV-1 residue 119469. The corresponding region of HSV-2 is on the vertical axis. The unpublished HSV-2 sequence was kindly supplied by Dr. Duncan McGeoch. The comparison indicated a series of short conserved loci, and are numbered on the panel from 1 through to 9.

COMPARE Window: 21 Stringency: 16.0 Points: 1,628

Hsv2newr1.Seq ck: 6,228, 5,000 to 6,600



Hsv1lat.Seq ck: 4,365, -1462 to 138

Figure 7. Alignment of HSV-1 and HSV-2 sequences upstream of the 2 kb LAT specifying region (three pages). This is an extended version of a sequence alignment generated by Dr. Duncan McGeoch using the GCG program Gap. The HSV-1 sequence is shown on the upper line, the HSV-2 sequence on the middle line and the consensus on the lower line. HSV-1 sequence numbering is with respect to the position of the mapped 5' end of the 2 kb LAT which is indicated at position 0. The locations of the consensus loci identified in Figure 6 are indicated by dotted lines underneath the consensus. Putative promoter elements identified in the HSV-1 sequence are indicated in bold letters, and labelled above the HSV-1 sequence. The positions of three ATG codons in the HSV-1 sequence downstream of the TATA box are indicated by bold lettering.

TTATGTGGCCGCGG**CCAGCAG**CAGAGCGGCCGCG.....ACACCCCC**CCCGCCCG**GTGTG.....GATATCCG -868
TTACGTGGCCGCGG**CCAGCAG**CAGAGCGGCCGCGG**CCAGCAG**ACGAGAGCGGCCGCGCCAGCGCCACGCTCCCGCCATTAGCCCCCGGAGCATCCG 5670
TTA-GTGGCCGCGG**CCAGCAG**CAGAGCGG-CCGCG-----C-C-C-CC-CCCGCC---T-----GG-ATCCG
-- Conserved Locus 3 ----->
<-----

GCCCC**CCGCGCCGCG**GTCCATTAAAGGGCGCGGTGCCCGCAGATAT**CAAT**CCGTTAAGTCTCTGCAGACAGGGCACCGCCCGCCGGAATCCATT -768
GCGCCCGCC**CCAGCAG**CGCCCTTCCATTAAACTTCCACGTTGGGGGGGGCCCGCCAGCTGAGTGTCTCTGCGTTGCGCGCCGCGCCGAGATCCATT 5770
GC-CC-GCCCC-CGCC--TCCATTAA---C-C-C--G---G-G-----C-G-T-AGTGTCTGTG-C---GGGC-CCG-GCCCGGA-ATCCATT
- Conserved Locus 4 ----->
<----- Conserved Locus 5 -----

AGGCCGACAGCAGGAAATTAATAATTACATTCACCTAAC**CCACG**GTGGTGTGCTGTGGCCCTGTTTTTGC**TCCGTCA**TCTCAGCC**TTTATA**AAAGCGGGGCGCGG -668
AAGCCCGCGGAGAGCCCGAGC.....CCCCCGCGTGTGTGCTGTGGC.ATTTCTGCTGCGTCA**TCCCTGTCTT**ATAAAACCGGGGGCGCGG 5858
A-GCCGC-G--GAG-----A-----CC--CCC-CGTG-TGCTGTGG-C--TTT-TGCTGCATC-C-G-CTTTATAAA-CGGGGGCGCGG
----->
<----- Conserved Locus 6 -----

CCGT.....GCCGATCGCGGGTGTGCGAAAGACTTCCGGGCGCGTCCGGGTCCCGGCTC**CCGGCC**CCCGCCCTGCAGCCGGG -588
CAGCAACGAACGACAGGGCGCCCGCGATCGAGAGGGACTCCGGAGAAAGAGCTGCTCCGGCACCGGGCGCCCTTCTCCTCCCTCCCTACCT 5958
C-G-----GCCGATCG-G-G--C---AGA-----G-----CG-----GC-CT-C---C-CC-C-----
>

GCGCCAAAGGGCGTGGCGACATCCTCCCTTAAGCGCCGGCGCGCTGGTCTGTCTTTTTCGTTTTCCCGTTTCGGGGGTGGGGTTCGGTT -488
CCCCCTCTCTTCCCCCTTTTTCCCCGCCCTCCCGTCTTCTCCGCGCTCCGAGGGTCCCGCTTCCCTCCGGGACCCCGGGCGGGGCTTGG 6058
-C--C-----C--C-----CC-CC-C-----C--C--CCG--C-----G--GT-----TT--C-C-G---C-----G-G--GG--G--GC----

TCTGTTTCTTAAACCCTGTCGGGGTGTTTTTCGTTCCGTCGCCGGA**AT**GTTCGTTCTGTCGCCCTCACGGGGCGAAGCCCGCTACGGCCCGGGACG
CCGCCGAG.....GTGGCCCGCCGGCGG
-C-----GT-CG-CCCGG---G
<-----

AGGGCCCCCGC.ACCGGCGGGTCCCGGGCCCGTCGCCGACCCGCTCGCCGGCACCGCAGCGGAA**AA**AGCCCCCGGAGGCTTTTCCGGTTCCCGCC
AGGGCCCCCGCACCTCGGGCC..GCCCCCTCCGGCCCGCGCTTCGGAAAGCGCGAAAGGGCCCCCG.GGAGGCTTTTTCGATTCCCGCCG
AGGGCCCCCG-ACC-CGGCGG-C---GCCCC-TCCGG--CCGC-CG---GC---G-CGGAAA--GGCCCC-GGAGGCTTTT---G-TTCCCGGCC-
Conserved Locus 7 ---> <----- Conserved Locus 8 -----

GGGGCCTGAG**AT**GACACTCGGGTTACCGCCAAAGCCCGCCCGCTGGCCGGCCCGCCCGGGCCCGCGGACCCAAAGGGCCCC.GGCCCGGGC
GGGGTCCCGGTAGCCCGCCCGCGCGGGGGAAGCGTCCCGCCCGCGGGTCCGGCCCGGGCCCGCGGAGCGCGGGCCCGGGCCCGGGCCCGGGC
GGGG-C---G-T---C-C-CGG-G-----AA-G-----CCC-C---GGCGG-CCGGCCCGGG-C---GGGGCCCC-GGCC-GGGC
---> <----- Conserved Locus 9 -----

CCCAACAAGCCCGCGC**AT**GCCTGTGGTTTTTTTTTCCCTCGGTTCTGCCCCGCTCCATCGCCCTTTCCTGTTCTCGCTTCCCC.CCCCCCTTCT
CGCGC.CGG.CGGCGTTTCCCGCGTTCGGTTTCTTCCCTCCCGGGCCCGCCCGCTCCCGGGCCCGAC...CTCGCCCTTCCCTTCTCGCT
C-C-C--CGG--CGCG--T-CGC--T---TTT--TT--TCC-----G--C-GCC--GCTCC--GCC---CC---CTCGC--CT-CC-C-CT
>

TCACCCCCAGTACCCCTCCCTCCCT..TCCT...CCCCCGTTATCCCACTCGTCGAGGGCCCGCCCGGTGTCGTTCAACAAGACCGCCGCTTTCAG
TCCCCCGTCCCGCGCCCTTCCCTTCTTCTCTCTCTCTGTCGCTCCCTACATTTCCCCCCCCCGCCCGCCCGCCCTTTGGCCCGC
TC-CCC-----CC---C-CC-TCCCT--TCCT---C-C-C--T-TC-C-CTC-TC-----CCCC-----C---C---G-CGCC--T--CC--

-5' end 2 kb LAT --->
GTAGGTTAGACAC.CTGCTTCTTCCCAATAGAGGGGGGGAACCCAAACGACAGGG
GTCCCAACGAGACCGCCCGCGCTGAGCCGTCGCCGGGGGAACCAAGCCTCCGG
GT-----GA-A--C-GC--G-C---A---G---GGG---C--A-G-----GG
0
54
6451

homologue. Both of the HSV-1 putative Sp1 binding sites harbour single base pair substitutions in the HSV-2 sequence: The proximal Sp1 site at -864 to -859 located in conserved locus 4 has a single 5'G replacing a C residue (...GCCGGCCCCA...) while the distal Sp1 site at position -887 to -882 has the 3'C replaced by a G residue (...TCCCGCCGGC...).

The occurrence of this putative RNA polymerase II promoter within a region of significant HSV-1/HSV-2 homology suggested that these sequences were of functional significance. The most logical conclusion was that they were involved in transcriptional control of the LAT gene. The TATA box may form the central feature of a core promoter, the activity of which is enhanced by upstream sequences (the CAAT and Sp1 binding sites perhaps), or perhaps by other sequences located around conserved loci 1 to 9.

3B.4. Identification of Neuronal Specific Sequences within the LAT Region

Since tissue specific expression of many genes is often governed by *cis*-acting elements located within their 5' flanking region, and LAT gene expression appears to be relatively neuronal specific, a comparison of the LAT 5' flanking region with the flanking regions of neuronal specific cellular genes may well reveal the presence of interesting sequence homologies. While studies have yet to reveal sequence elements responsible for directing neuronal specific gene expression, homologous sequences in the 5' flanking regions of genes expressed in brain and PC12 neuroblastoma cells which may be of general importance for neuronal-specific expression of proteins have been identified.

Maue *et al*, (1990), when comparing the 5' flanking regions of the rat type II sodium channel gene with other neuronal specific genes whose levels of expression are

similarly increased during NGF treatment of PC12 cells, identified a short region of homology based around a core 5'-CCAGG-3' consensus motif. The sequence of this region of the type II sodium channel together with the homologous regions of the rat peripherin gene containing two separate sequences with 92% and 100% identity, the mouse neurofilament gene (77% identity), and the *drosophila* dopa decarboxylase gene (86% identity) are displayed on Figure 8. In the case of the *drosophila* dopa decarboxylase gene, this element has been shown to be necessary but not sufficient for expression in the central nervous system of *drosophila*. (Scholnick *et al.*, 1986; Johnson *et al.*, 1989). While the homologues among the other neuronal specific genes are intriguing, it has yet to be determined whether they are of functional significance. Analysis of the HSV-1 LAT sequence reveals two potential homologues (see Figure 8) of this motif at -915 to -911 (...CCAGC..) and -731 to -727 (...CCACG..). The former sequence shares 81% identity (9/11 bases; from -915 to -905) with the type II sodium channel and occurs in a region of high homology (conserved locus 3) with the HSV-2 sequence. The latter sequence shares 12/14 bases; from -731 to -718 with a gap, with the sequence of the type II sodium channel gene, and while this also occurs in a region of HSV-1/HSV-2 homology (conserved locus 6), the actual sequence around the core homology is less well conserved. This shows that the HSV sequences are homologous to the neuronal sequences as the neuronal sequences are to each other. Analysis of the rest of the HSV-1 sequence failed to find any other significant homologues. The identification of these two sequences which bear striking resemblance to a consensus possibly involved in transcriptional regulation of neuronal genes is intriguing, and it is interesting to note that Zwaagstra *et al.*, (1991) have identified a LAT promoter binding factor (LPBF), present in neuronal and non-neuronal cell lines, which binds to a palindromic sequence based around the

Figure 8. Homology between two regions of the putative LAT promoters of HSV-1 and HSV-2, and the 5' flanking sequences of neuronal specific genes. The rat type II sodium channel sequence is between nucleotides -60 and -34. The *drosophila* sequence is between nucleotides -61 and -72 on the opposite strand. The rat peripherin sequences represent nucleotides -163 to -154 and -72 to -62 respectively. The mouse neurofilament sequence is from -73 to -62. The HSV-1/HSV-2 homologues are shown below, and refer to nucleotides -921 to -899 (-262 to -240 with respect to the 5' end of m-LAT) and -737 to -716 (-78 to -57) of the HSV-1 sequence respectively. The CCAGG consensus identified by Maue *et al.* (1990) is indicated on the top line.

CCAGG
ACTTGTGACCAGGAGATGGAGCTGTCG
ACTTG G CCAGGA
ACCAGGAGAGGGA
CCAGG TGGAGCCGCAG
CGCGGGCCAGCAGACGGCCCGCG
CGCGGGCCAGCAGACGG CCGCG
ACCTACCCACG TGGTGCTGTGG
CCCGCCCGCG TGTGCTGTGG

CCACG TGG

Core consensus motif
Rat type II sodium channel
Drosophila dopa decarboxylase
Rat peripherin
Mouse neurofilament
HSV-1 LAT (-921 to -899)
HSV-2 LAT
HSV-1 LAT (-737 to -716)
HSV-2 LAT

LPBF motif

CCAGG homologue; "CCACGTGG"; located at -731 to -724. Gel shift assays with a probe containing the distal CCAGG homologue did not, however, identify any prominent bands.

3C. ANALYSIS OF THE PUTATIVE LAT PROMOTER IN TRANSIENT EXPRESSION ASSAYS

3C.1. Strategy

In order to physically characterise the putative LAT promoter region, various sequences around and upstream of the mapped 5' end of the 2 kb LATs were cloned and examined for their ability to drive expression of an adjoining reporter gene in transient expression assays. This work is presented in four sections. The first three will concentrate on the 1141 bp region between the *Hin*FI site at -940 and *Nae*I site at +201 and encompassing conserved loci 3 to 9. These sections examine the effects on promoter activity of removal of sequences from the 5' end, the 3' end and the centre of this region respectively. The fourth section examines the effect of addition of an upstream region extending back to the *Dra*I site at -1459 which contains consensus loci 1 and 2. These sequences, together with conserved loci and restriction sites are displayed on Figure 9. Since reference will constantly be made to this sequence and its restriction sites, for ease of reference, it is provided in pull-out form in the back cover of the thesis.

3C.2. Deletions from the 5' End

2.1. Cloning of Putative Promoter Fragments

In order to examine the effect on promoter activity of removal of 5' sequences from the 1141 bp *Hin*FI-*Nae*I region, a series of DNA fragments were cloned into the chloramphenicol acetyltransferase (CAT) containing vector pFJ5 (Rixon & McLauchlan, 1990; see Figure 10). These

Figure 9. Nucleotide sequence of the 5' flanking region of the 2 kb LAT (next two pages). The 5' end of the 2 kb LAT is marked at position 0. The locations of the conserved loci identified in Figure 6 are indicated by dotted lines underneath the sequence. Putative promoter elements are indicated by bold lettering and labelled above the sequence, as are the positions of all of the restriction enzyme sites used in construction of clones for characterisation of the LAT promoter. The G immediately downstream of the TATA box was found to be changed to an A when one of the clones constructed for LAT promoter analysis was sequenced (see Figure 16). This Figure is also provided in the back cover of this thesis.

DraI

-1461 **TTTAAA**TAAACCAATGTCGGAATAAACAAACACAAACACCCGCGACGGGGGGACGGAGGG

-1401 GACGGAGGGAGGGGGTGACGGGGGACGGGAACAGACACAAAAACAACCACAAAAACAAC

-1341 CACCCACCGACACCCCCACCCAGTCTCTCGCCTTCTCCCACCCACCCACGCCCCAC

-1281 TGAGCCCGGTTCGATCGACGAGCACCCCGCCACGCCCCCGCCCTGCCCGGGACCCC

-1221 CGGCCCGCACGATCCCGACAACAATAACAACCCCAACGGAAAGCGGCGGGGTGTTGGGGG

-1161 AGGCGAGGAACAACCGAGGGGAACGGGGGATGGAAGGACGGGAAGTGAAGTCTTGATA
 <----->

-1101 CCATCCTACACCCCCCTGCCTTCCACCCTCCGGCCCCCGCGAGTCCACCCGCCGGCCGG
 ----- Conserved locus 1 -----> <----->

-1041 CTACCGAGACCGAACACGGCGGCCCGCCGAGCCGCCGAGCCGCCGCCGACCCGCAGAG
 -- Conserved locus 2 --->

-981 CCGGCGCGGCGACTCACAAGCGGCAGAGGCAGAAAGGCC**AGAGTC**ATTGTTTATGTGGC
 <----->

HinfI

Sp1

-921 CGCGGG**CCAGC**AGACGGCCCGGACACCCCCCCCC**CCGCCC**GTGTGGGTATCCGGCCCCC
 - Conserved locus 3 --> <----->

Sp1

CAAT

-861 **GCCCCGCGCCGGTCCATTAAGGGCGCGCGT**CCCCGCGAGATAT**CAAT**CCGTTAAGTGCTC
 Conserved locus 4 -> <----->

PstI

-801 **TGCAGACAGGGGCACCGCGCCCGGAAATCCATTAGGCCGAGACGAGGAAAATAAAATTA**
 ----- Conserved locus 5 ----->

LPBF

CRE

TATA

-741 CATCACCTACCC**CACGTGG**TGCTGTGGCCTGTTTTGCT**TGCGTC**ATCTCAGCC**TTTATAAA**
 <-----> Conserved locus 6 ----->

PvuI

-681 **AGCGGGGGCGCGCCGTGCCGATCG**CGGGTGGTGCGAAAGACTTTCCGGGCGCGTCCGGG
 ----->

PstI

-621 TGCCGCGGCTCTCCGGGCCCC**CTGCAG**CCGGGGCGGCCAAGGGGCGTCGGCGACATCCT

NaeI

-561 CCCCCTAAGC**CGCGCC**CGGCGCTGGTCTGTTTTTCGTTTTCCCGTTTTCGGGGGTGGT

-501 GGGGGTTGCGGTTTCTGTTTCTTTAACCCGTCTGGGGTGTTTTTCGTTCCGTCGCCGGAA

-441 TGTTTCGTTTCGTCTGTCCCCTCACGGGGCGAAGGCCGCGTACGG**CCCCGGG**ACGAGGGGCC
SmaI
<----- Conserved

-381 CCCGACCGCGGGCGGTCCGGGCCCGTCCGGACCCGCTC**GCCGGC**ACGCGACGCGAAAAAG
NaeI
locus 7 -----> <----->

-321 GCCCCCGGAGGCTTTTCCGGGTTCCCGGCCCGGGCCTGAGATGAACACTCGGGGTAC
---- Conserved locus 8 ----->

-261 CGCCAACG**GCCGGC**CCCCGTGGCGGCCCGGCCCGGGGCCCGGGCGGACCCAAGGGGCCCC
NaeI
<----- Conserved locus 9 ----->

-201 GGCCCGGGGCCCAACAACGGCCCGGG**GCATGC**GCTGTGGTTTTTTTTTCTCGGTGTTCT
SphI
----->

-141 GCCGGGCTCCATCGCCTTTCTGTTCTCGCTTCTCCCCCCCCCTTCTTACCCCCAGTA

-81 CCCTCCTCCCTCCCTTCCCTCCCCGTTATCCCACTCGTCGAGGGCGCCCCGGTGTCGTTT

|-- 5' end of 2 kb LAT --->

-21 AACAAAGACGCCGCGTTTCCAG**GT**AGGTTAGACACCTGCTTCTCCCAATAGAGGGGGG

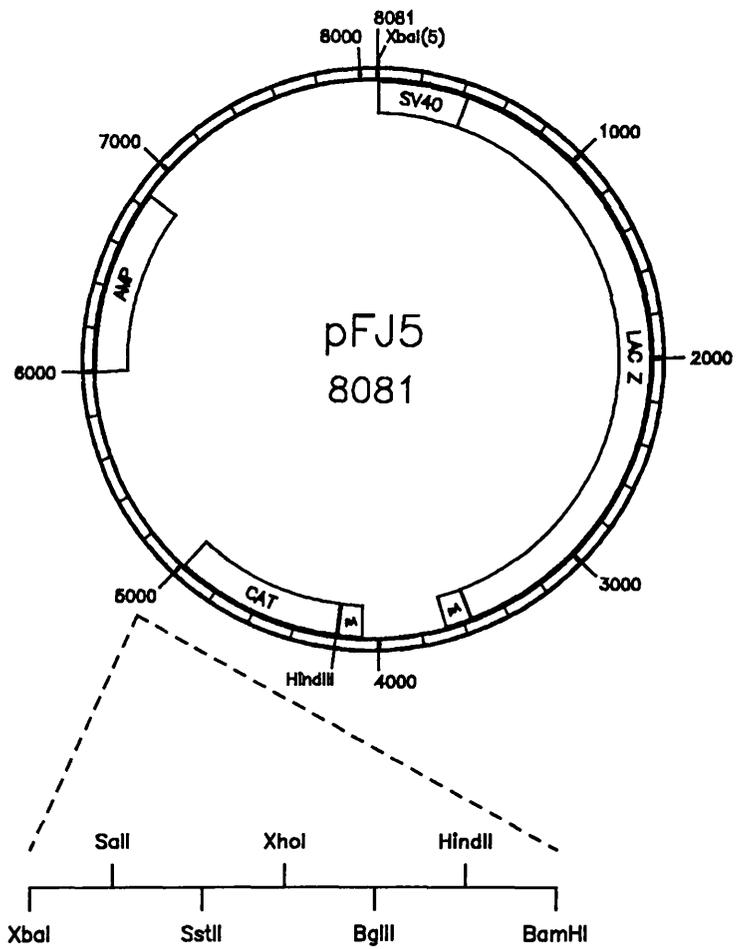
39 GACCCAAACGACAGGGGGCGCCCCAGAGGCTAAGGTCGGCCACGCCACTCGCGGGTGGGC

99 TCGTGTTACAGCACACCAGCCGTTCTTTTCCCCCCTCCACCCCTTAGTCAG**ACTCTGT**
HinfI

159 TACTTACCCGTCCGACCACCAACTGCCCCCTTATCTAAGG**GCCGGC**TGGAAGACCGCCAG
NaeI

219 GGGGTCGGCCGGTGTCGCTG

Figure 10. Structure of plasmid pFJ5. Derivations of this plasmid was used for characterisation of the LAT promoter in transfection assays. pFJ5 contains the *E.coli lacZ* gene driven by the SV40 early promoter and terminating with the SV40 polyadenylation sequences. The CAT gene is inserted immediately downstream of a polylinker sequence (shown below the plasmid) and is terminated by the HSV-2 IE-5 polyadenylation sequence. A thorough account of the construction of pFJ5 is given in Rixon & McLauchlan (1990).



fragments are presented diagrammatically in Figure 11. The precise location of the various restriction enzyme sites can be found on Figure 9.

pLAT1CAT: pLAT1CAT contains a 1091 bp fragment which extends from -939 to +151 of the LAT sequence and contains all of the recognisable putative promoter elements. To construct pLAT1CAT, the 1091 bp *HinfI*-*HinfI* fragment was excised and purified from plasmid pGX48 which consists of the HSV-1 *BamHI* *b* restriction fragment cloned into the *BamHI* site in pAT153. This *HinfI*/*HinfI* fragment was subsequently blunt-ended with T4 DNA polymerase and ligated into *BamHI*-digested, T4 DNA polymerase-treated pUC19 to generate plasmid pLAT1. The *EcoRI* site present in the pUC19 multicloning site of pLAT1 was converted to a *BglII* site by *EcoRI* digestion, T4 DNA polymerase treatment and subsequent ligation of a phosphorylated *BglII* linker 5'-CAGATCTG-3', thus yielding plasmid pLAT1+*BglII*. This treatment regenerated *EcoRI* sites on either side of the *BglII* linker insertion. The LAT1 sequence was excised from pLAT1+*BglII* as a *Sall*/*BglII* fragment and subsequently ligated into *Sall*/*BglII* digested pFJ5 DNA to generate pLAT1CAT.

pLAT2CAT: pLAT2CAT contains 949 bp of LAT sequence which extends from the *PstI* site at -798 to the *HinfI* site at +151, and is deleted for the CAAT and Sp1 homologies. These sequences were cloned from pJR3 which consists of the *SstI*/*PstI* fragment from IRL (spanning the complete IE-1 gene to the *PstI* site at -798 of the LAT sequence) cloned into pUC9. To construct pLAT2CAT, pJR3 was digested with *HindIII* and *HinfI*; the 950 bp fragment was purified and blunt-ended with T4 DNA polymerase. This fragment was ligated into *XhoI*-digested and T4 DNA polymerase-treated pFJ5 to generate pLAT2CAT.

pLAT3CAT: pLAT3CAT contains 746 bp of LAT sequence which

spans from the *Pst*I site at -595 to the *Hinf*I site at +151 and does not contain any of the putative LAT promoter elements. To construct pLAT3CAT, pJR3 (described above) was digested with *Pst*I and *Hinf*I. The 746 bp fragment was purified, blunt-ended with T4 DNA polymerase and ligated into *Bam*HI digested/T4 DNA polymerase treated pUC19 DNA to generate pLAT3. The *Sst*I site in the pUC19 polylinker of pLAT3 was converted to a *Bgl*III site by *Sst*I digestion and T4 DNA polymerase treatment followed by ligation of the phosphorylated *Bgl*III linker 5'-CAGATCTG-3' to generate plasmid pLAT3-*Sst*I+*Bgl*III. The LAT3 sequence was excised from pLAT3-*Sst*I+*Bgl*III as a *Sal*I/*Bgl*III fragment and ligated into *Sal*I/*Bgl*III digested pFJ5 DNA to generate pLAT3CAT.

pIF5: pIF5 contains 402 bp of LAT sequence spanning from the *Nae*I site at -251 to the *Hinf*I site at +151. To generate pIF5, pLAT1CAT was digested with *Sal*I and *Nae*I, blunt-ended with T4 DNA polymerase and religated.

pLAT5CAT: pLAT5CAT contains 452 bp of LAT sequence which spans the *Nae*I sites at -251 and +201. To construct pLAT5CAT, pJR3 (described above) was digested with *Nae*I. The appropriate 452 bp fragment was purified, blunt-ended with T4 DNA polymerase and ligated into *Bam*HI and T4 DNA polymerase treated pUC19 DNA to generate pLAT5. The *Eco*RI site in the pUC19 polylinker of pLAT5 was converted to a *Bgl*III site by *Eco*RI digestion, followed by blunt-ending with T4 DNA polymerase and subsequent ligation of the *Bgl*III linker 5'-CAGATCTG-3' to generate pLAT5+*Bgl*III. The LAT5 fragment was excised from pLAT5+*Bgl*III as a *Sal*I/*Bgl*III fragment and subsequently ligated into *Sal*I/*Bgl*III digested pFJ5 DNA to generate pLAT5CAT

Test restriction digests demonstrating the integrity of the above LATCAT plasmids, together with those described in preceding sections are shown on Figures 12 and

13. Other constructs used as controls in the subsequent transfection assays are as follows:

pFJ5: Negative control plasmid which does not have any promoter driving CAT activity.

pFJ10: Has the promoter for the HSV-2 IE 4/5 promoter fused to the CAT gene in pFJ5. (Rixon & McLauchlan, 1990).

pgDCAT: Has the promoter for the glycoprotein D (gD) gene of HSV-1 fused to the CAT gene in pUC9 (Everett, 1986).

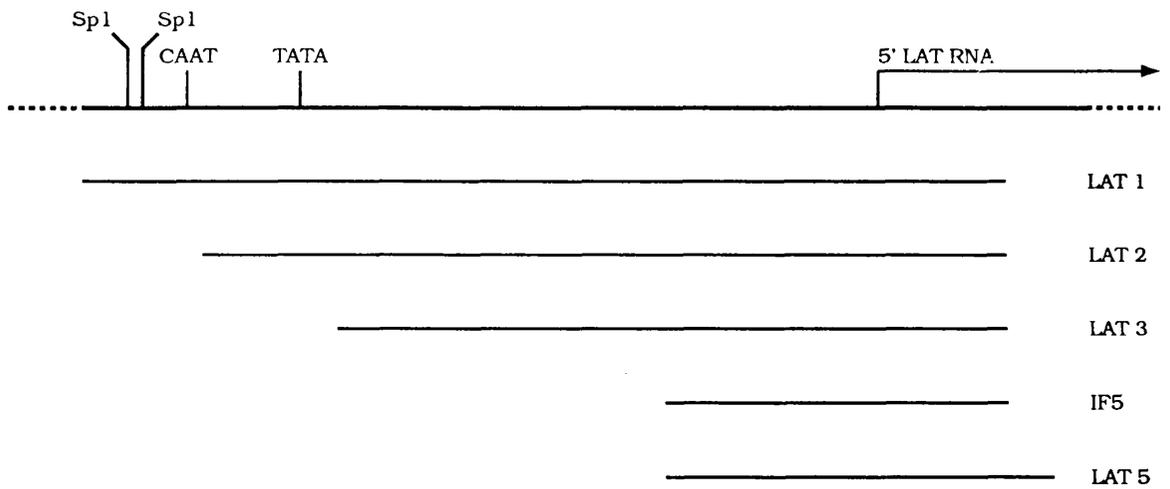
Plasmids pFJ10 and pgDCAT contain examples of HSV immediate-early and early genes respectively, and are normally active during the lytic stage of HSV infection.

2.2. Expression Assays Using LATCAT Plasmids

The promoter activity of the above constructs were determined by quantitation of CAT activity in short term transfection assays. 3 μ g of each plasmid was transfected into BHK monolayers using a modified version of the calcium phosphate technique used by Shen *et al.* (1982). CAT extracts were made and assayed for CAT activity as described by Seed & Sheen, (1988). The results illustrated in columns 2 and 3 of Table 3 give the mean of at least four independent determinations along with the standard error of the mean. These, and all other results from transfection experiments were calculated relative to the promoter activity of pLAT1CAT which was included in every experiment. The CAT activities from each transfection experiment were calculated as percentage conversion per hour per μ g protein, and then normalised relative to the CAT activity exhibited by pLAT1CAT whose activity was assigned the nominal value of 1. An example of this calculation using original data is shown on Figure 14. This

Figure 11. Schematic representation of LAT promoter fragments with deletions from the 5' end. The top line indicates the relative positions of the putative promoter elements and 5' end of 2 kb LAT. The LAT1 fragment extends from -940 to +154, the LAT2 fragment from -798 to +154. the LAT3 fragment from -595 to +154, IF5 from -251 to +154, and LAT5 from -251 to +201.

Table 3. Relative CAT activities exhibited by LAT promoter plasmids. The CAT activities exhibited by each of the LAT promoter plasmids, along with controls pFJ5, pgDCAT and pFJ10 were determined and calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1. The mean of at least four independent determinations is given (column 2) with the standard error of the mean (SEM) (column 3). Column 4 records the relative CAT activity exhibited upon infection of transfected cells with 5pfu/cell HSV-1 17+ for 18h, with the SEM given in column 5. The level of induction of CAT activity upon infection, relative to uninfected cell CAT activity is given in column 6.



| Plasmid | Relative CAT Activity | | | | Induction |
|----------|-----------------------|------------------|----------|------------------|-----------|
| | Uninfected | Std. Error (+/-) | Infected | Std. Error (+/-) | |
| pFJ5 | 0.11 | 0.04 | 0.92 | 0.15 | 8.3 |
| pFJ10 | 48.32 | 8.5 | 612.3 | 89.59 | 12.7 |
| pgDCAT | 4.4 | 0.79 | 534.9 | 94.8 | 121.56 |
| pLAT1CAT | 1 | 0.28 | 3.69 | 0.54 | 3.69 |
| pLAT2CAT | 0.14 | 0.02 | 1.52 | 0.21 | 10.8 |
| pLAT3CAT | 0.02 | 0.009 | 0.37 | 0.12 | 15.8 |
| pIF5 | 0.38 | 0.17 | 3.57 | 0.43 | 9.39 |
| pLAT5CAT | 0.04 | 0.02 | 4.5 | 1.85 | 112.5 |

Figure 12. Restriction digest profiles of LAT promoter plasmids. Plasmids pLAT1CAT (lane 1) to pLAT7CAT (lane 7) were digested with *Hind*III and *Xba*I and run on a 1% agarose gel along with 123 bp ladder size markers (lane S). The top two fragments in each of the plasmid lanes represent vector sequences. The third fragment co-migrating in all tracks is the 797 bp *Hind*III fragment containing the CAT gene. The *Hind*III/*Xba*I LAT promoter fragments marked (P) all migrate distances consistent with their expected sizes. Note that the LAT3 promoter fragment co-migrates with the CAT fragment.

Lane S: 123 bp ladder

Lane 1: pLAT1CAT

Lane 2: pLAT2CAT

Lane 3: pLAT3CAT

Lane 4: pLAT4CAT

Lane 5: pLAT5CAT

Lane 6: pLAT6CAT

Lane 7: pLAT7CAT

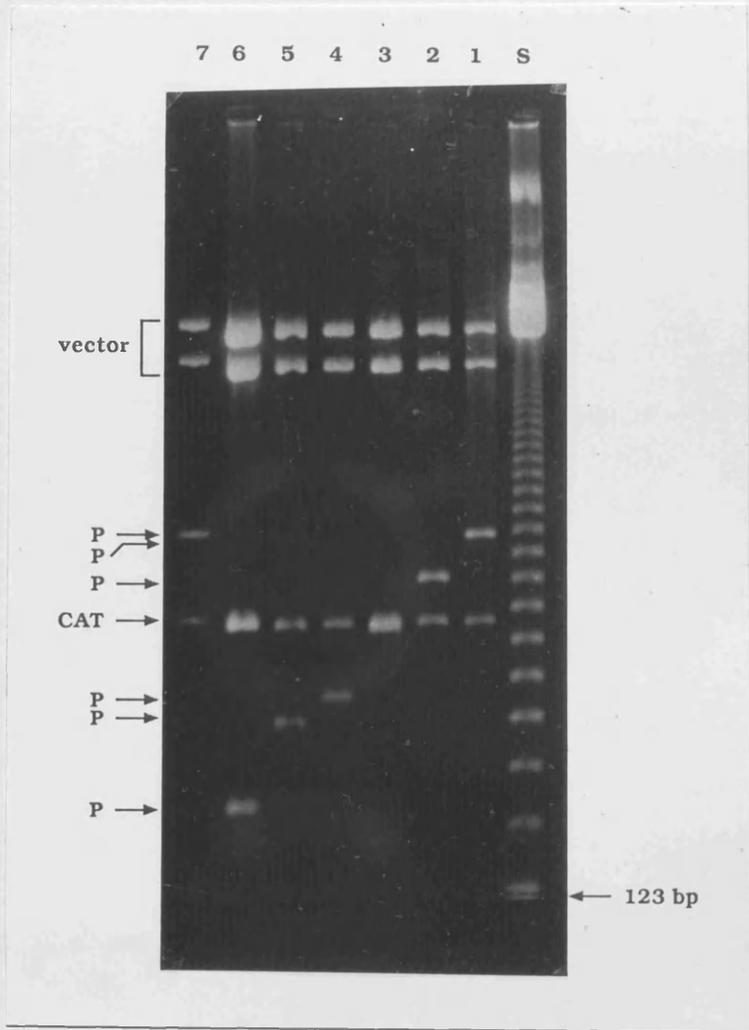


Figure 13. Restriction digest profiles of LAT promoter plasmids. The plasmids indicated were digested with *HindIII* and *XbaI* and run on a 1% agarose gel along with 123 bp ladder size markers (lane S). The top two fragments in each of the plasmid lanes represent vector sequences. The third fragment co-migrating in all tracks is the 797 bp *HindIII* fragment containing the CAT gene. The *HindIII/XbaI* LAT promoter fragments marked (P) all migrate the correct distances consistent with their expected sizes.

Lane S: 123 bp ladder

Lane 1: pLAT8CAT

Lane 2: pIF5

Lane 3: pLAT4/5CAT

Lane 4: p Δ 1CAT

Lane 5: p Δ 2CAT

Lane 6: p Δ 3CAT

Lane 7: pLAT0CAT

Lane 8: pLAT60CAT

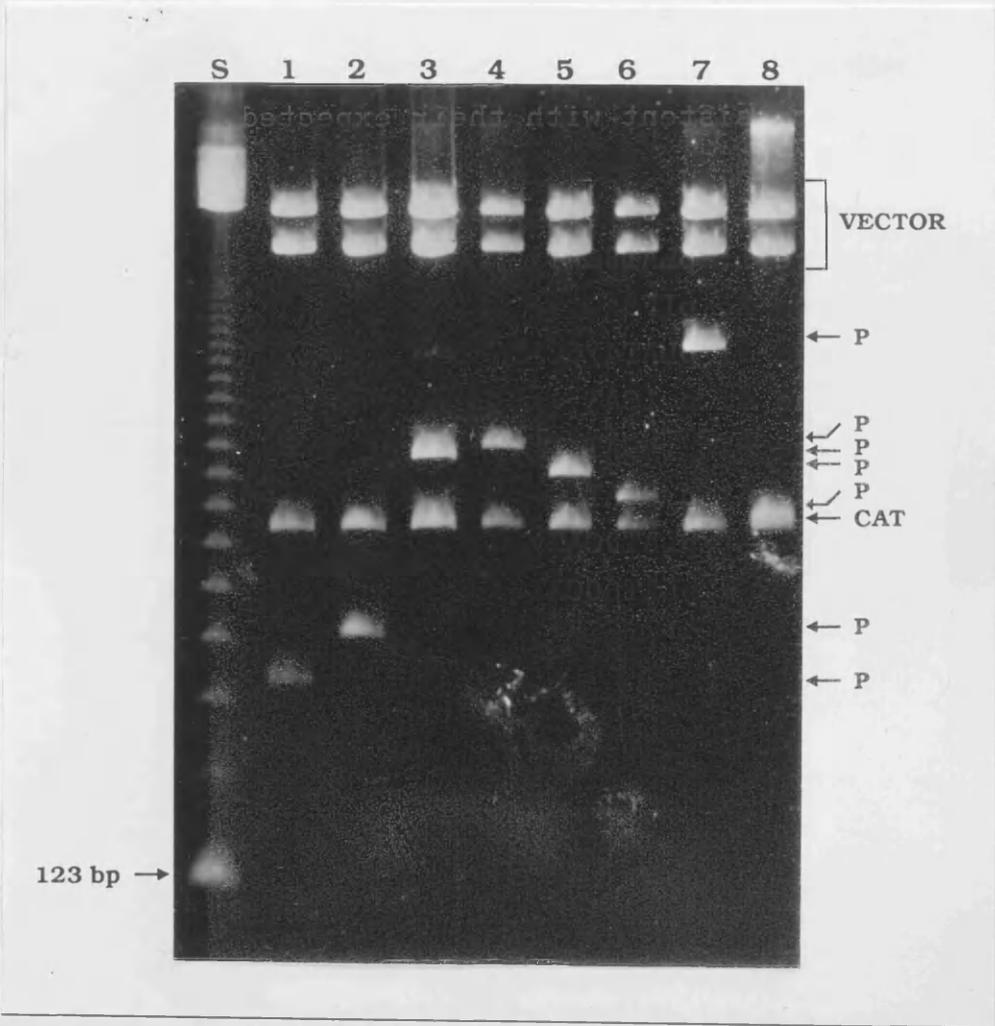


Figure 14. CAT Activity: Original data sample. This gives an example of how the relative CAT activities from the transfection assays were calculated from original data. CAT assays were carried out as described by Seed & Sheen (1988). Reactions were carried out for 1h at 37°C, then extracted with a 2:1 mixture of TMPD/Xylenes. The cpm from the organic phase (O) and aqueous phase (A) of a control (blank) reaction were determined. Percentage conversion per h per μg protein, was calculated as shown. The CAT activities of all of the plasmids were finally calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1.

CAT ACTIVITY: ORIGINAL DATA

Organic phase counts (O): 128.

Aqueous phase counts (A): 98,452.

| | counts per min. (extract diln) | | Protein conc. | |
|------------|--------------------------------|--------------|---------------|-----|
| | UI | I | UI | I |
| pFJ5 | 263 | 2253 | 380 | 95 |
| pgDCAT | 4000 (x10) | 8817 (x600) | 240 | 85 |
| pFJ10 | 8271 (x100) | 11604 (x600) | 230 | 80 |
| pLAT1CAT | 1676 (x10) | 2868 (x10) | 200 | 85 |
| pLAT2CAT | 3844 | 1494 (x10) | 210 | 85 |
| pLAT3CAT | 482 | 5943 | 220 | 110 |
| pLAT4CAT | 1820 (x10) | 2665 (x20) | 200 | 125 |
| pLAT5CAT | 305 | 6543 (x10) | 145 | 125 |
| pLAT6CAT | 1979 (x20) | 6468 (x100) | 145 | 110 |
| pLAT4/5CAT | 2652 (x10) | 4021 (x400) | 185 | 100 |

% conversion/h = [(cpm-O)/A]x100

%conversion/h/ μ g protein = %conversion/protein conc.

| | % conversion/h/ μ g protein | | Normalised for pLAT1CAT=1 | |
|------------|------------------------------------|-------|------------------------------|--------|
| | UI | I | UI | I |
| pFJ5 | 0.0003 | 0.022 | 0.004 | 0.028 |
| pgDCAT | 0.164 | 62.25 | 2.09 | 792.9 |
| pFJ10 | 3.596 | 87.37 | 45.8 | 1113.1 |
| pLAT1CAT | 0.078 | 0.327 | 1 | 4.16 |
| pLAT2CAT | 0.017 | 0.162 | 0.228 | 2.06 |
| pLAT3CAT | 0.001 | 0.054 | 0.020 | 0.687 |
| pLAT4CAT | 0.086 | 0.411 | 1.09 | 5.23 |
| pLAT5CAT | 0.001 | 0.520 | 0.015 | 6.62 |
| pLAT6CAT | 0.259 | 5.850 | 3.30 | 74.5 |
| pLAT4/5CAT | 0.138 | 15.80 | 1.75 | 201.3 |

calculation ^{standardises the} variability between different sets of CAT assays arising from the ^{variable} nature of the short term transfection assay which is due to factors such as transfection efficiency. The data from all of the transfection experiments were then pooled, and the mean and standard error calculated for each construct. The results presented for pLAT1CAT in Table's 3, 4, 5 and 6 were calculated only from data from experiments where the other constructs had been included, thereby explaining the slight differences in value for pLAT1CAT activities in each table.

As observed from the results on Table 3, the control plasmids pgDCAT and pFJ10 exhibited relatively high levels of constitutive activity, with pFJ10 being 11-fold higher than pgDCAT. PLAT1CAT exhibited a level of activity (about 23% of pgDCAT) which indicates that the LAT1 fragment contains sequences that can perform as a promoter in this assay. pLAT2CAT exhibits a 7-fold lower activity than pLAT1CAT while pLAT3CAT and pLAT5CAT exhibited negligible activity. The differences in activity between LATs 1, 2 and 3 indicate that the sequences important for promoter activity in LAT1 are located upstream of the *Pst*I site at -595 ie the region containing the recognisable putative promoter elements. It is therefore reasonable to conclude that the activity exhibited by the LAT2 fragment is due to the presence of the TATA box at position -689 to -681 which may form the basis of a core promoter, which is enhanced by upstream sequences between the *Pst*I site at -595 and *Hinf*I site at -940 (the CAAT box and putative *Sp*1 binding sites, perhaps) as in LAT1. It is not clear, however, why pIF5 exhibits such relatively high activity (about 38% of pLAT1CAT); the only difference between pIF5 and pLAT5CAT being additional 50 bp extension in pLAT5CAT.

2.3. Infection Studies on Promoter Activity

In addition to the transient expression assays

detailing constitutive promoter activity of the putative promoter clones, the effect on promoter activity of infecting the transfected cells with HSV-1 was investigated. The results from infection with wt HSV-1 are shown on columns 4 and 5 of Table 3, together with the level of induction above constitutive activity indicated in column 6.

Plasmids pgDCAT and pFJ10 exhibit induction to very high levels of CAT activity as would be expected for early and immediate-early promoters in response to HSV-1 *trans*-acting factors. The control plasmid pFJ5 also exhibits a response to infection, an effect probably due to the presence of cryptic promoters in the vector sequence. pLAT1CAT exhibits a relatively poor response to infection, being induced only 3.7-fold. pLAT2CAT was induced 11-fold to a level still less than that of induced pLAT1CAT. pLAT3CAT was induced 16-fold but yielded very little activity upon infection (cf pFJ5) thus confirming that this clone is devoid of sequences capable of functioning as a promoter in these assays. pIF5 was induced 3.57-fold to a level roughly similar to that of induced pLAT1CAT. Interestingly, LAT5 which yielded negligible constitutive activity, exhibited a 112.5-fold stimulation to a level greater than that of stimulated pLAT1CAT. It is clear from this result that LAT5 contains sequences which, though not capable of exhibiting any constitutive activity, respond strongly in the presence of HSV-1 *trans*-acting factors.

3C.3. Deletions from the 3' end of the LAT Region

The following section examines the effect on promoter activity of deletion of sequences from the 3' end of the 1141 bp *HinfI/NaeI* region of the LAT sequence. As in section 3C.2, a series of DNA fragments were constructed and cloned into pFJ5. The location of these fragments are diagrammatically represented in Figure 15.

3.1. Cloning of the Promoter Sequences

pLAT7CAT: Plasmid pLAT7CAT contains all 1141 bp of the LAT sequence between the *HinfI* site at -940 and the *NaeI* site +201. To construct pLAT7CAT, pLAT1 was digested with *SphI*, releasing a single *SphI* fragment spanning from the *SphI* site 5' to the LAT1 insert in the pUC19 polylinker to a *SphI* site at position -171 in the LAT sequence. This fragment was ligated into *SphI*-digested pLAT5+BglII DNA where the corresponding smaller *SphI* fragment had been removed, thus generating plasmid pLAT7. The LAT7 sequence was excised from pLAT7 as a *SallI/BglIII* fragment and ligated into *SallI/BglIII* digested pFJ5 DNA to generate plasmid pLAT7CAT.

pLAT4CAT: Plasmid pLAT4CAT contains 545 bp of LAT sequence spanning the *HinfI* site at -940 to the *SmaI* site at -395, and like pLAT8CAT and pLAT6CAT, contains all of the recognised putative promoter elements, but not the mapped 5' end of the 2kb LAT RNA, nor conserved loci 7, 8 or 9. To construct pLAT4CAT, pLAT1+BglIII was digested with *SmaI* and religated. This treatment deleted all of the LAT sequence between the *SmaI* site at -394 to the *SmaI* site in the pUC19 polylinker of pLAT1+BglIII, thus generating plasmid pLAT4. The LAT4 sequence was excised from pLAT4 as a *SallI/BglIII* fragment and ligated into *SallI/BglIII* digested pFJ5 DNA to generate plasmid pLAT4CAT.

pLAT8CAT: Plasmid pLAT8CAT contains 391 bp of LAT sequence encompassing the region between the *HinfI* site at -940 and the *NaeI* site at -549. To construct pLAT8CAT, pLAT1CAT was digested with *NaeI* and *BglIII*, blunt-ended with T4 DNA polymerase and religated. This treatment removed all of the LAT sequence from the *NaeI* site at -549 to the *BglIII* site in the pFJ5 polylinker in pLAT1CAT, thereby generating plasmid pLAT8CAT.

pLAT6CAT: Plasmid pLAT6CAT contains 279 bp of LAT sequence encompassing the region between the *HincII* site at -940 and the *PvuI* site at -659. To construct pLAT6CAT, pLAT1+BglIII was digested with *HincII* and *PvuI*. The 281 bp fragment was purified, blunt-ended with T4 DNA polymerase and ligated into *HincII* digested pUC8 DNA to generate pLAT6. The *Sall/BglIII* fragment of the appropriately orientated insert in pUC8 was isolated and ligated into *Sall/BglIII* digested pFJ5 DNA to generate plasmid pLAT6CAT.

3.2. The Sequence of pLAT6CAT

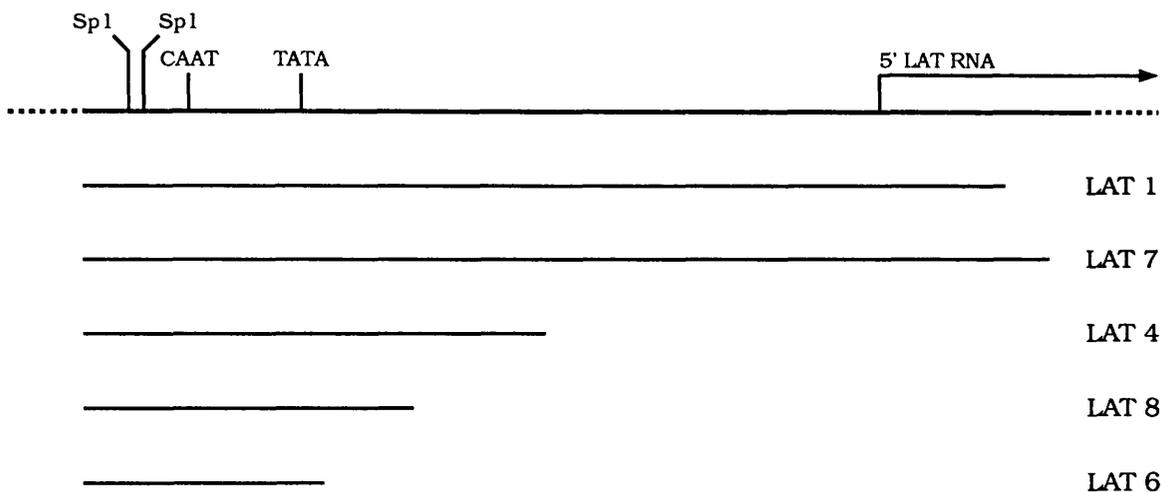
Since the LAT6 sequence includes all the putative promoter elements, it was sequenced to determine their integrity. This was performed by excising the LAT6 sequence from pLAT6CAT as a *Sall/BglIII* fragment and ligating it into *Sall/BamHI* digested pT7T3 19U. This enabled sequencing of the LAT6 sequence from either end of the clone. The sequencing gel, shown on Figure 16 indicates a single deviation from the published HSV-1 sequence. This is a base substitution at position -680 (G to A) which, despite its proximity to the TATA box, does not seem to affect the promoter activity exhibited by the LAT6 clone (see following sections). This position is also not conserved in the HSV-2 sequence with the G being substituted for a C, thereby suggesting that it is not crucial for LAT promoter activity. While not actually determined, it is likely that all of the other LAT clones harbouring this sequence will be the same except for perhaps pLAT2CAT which was independently cloned directly from pJR3 (section 3C.2.1).

3.3. Expression Assays

The promoter activity of the above constructs were determined by quantitation of CAT activity in short term transfection assays. The results are shown in columns 2 and 3 of Table 4. As before, the data were normalised relative

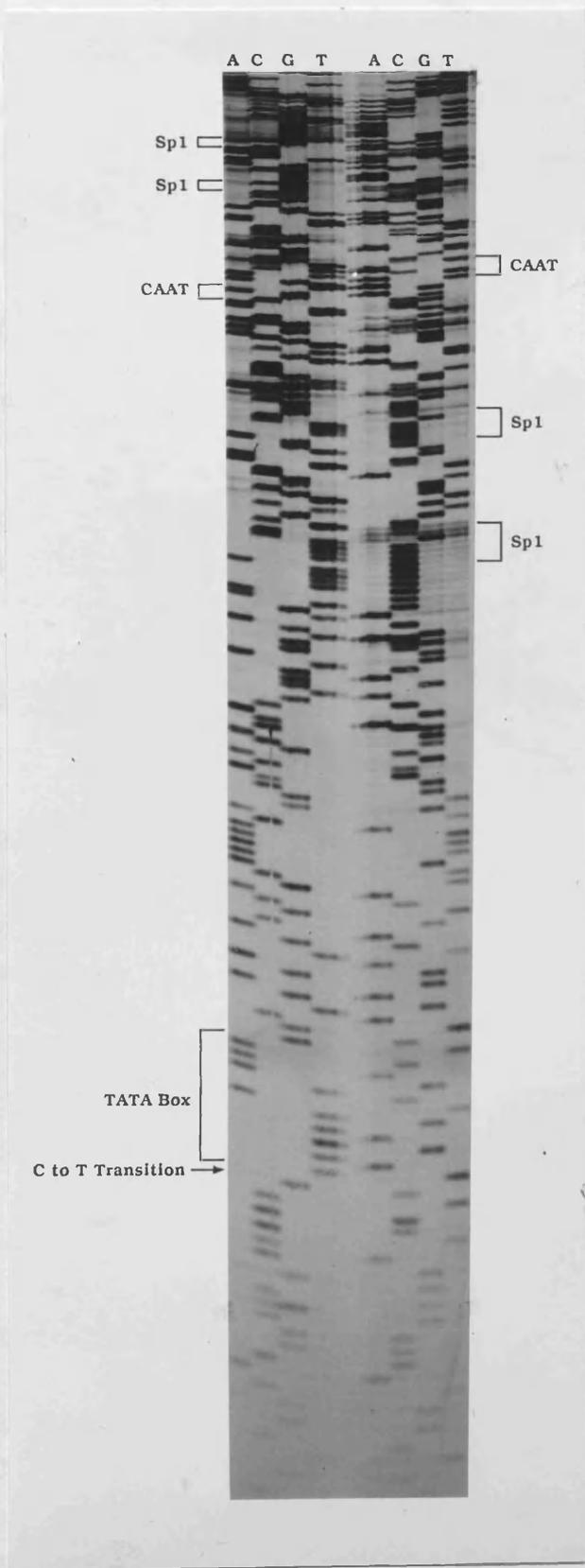
Figure 15. Schematic representation of LAT promoter fragments with increasing deletions from the 3' end. The top line indicates the relative positions of the putative promoter elements and 5' end of 2 kb LAT. The LAT1 fragment extends from -940 to +151, the LAT7 fragment from -940 to +201, the LAT4 fragment from -940 to -395, LAT8 from -940 to -549, and LAT6 from -940 to -659.

Table 4. Relative CAT activities exhibited by LAT promoter plasmids. The CAT activities exhibited by each of the LAT promoter plasmids were determined and calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1. The mean of at least four independent determinations is given (column 2) with the standard error of the mean (SEM) (column 3). Column 4 records the relative CAT activity exhibited upon infection of transfected cells with 5pfu/cell HSV-1 17+ for 18h, with the SEM given in column 5. The level of induction of CAT activity upon infection, relative to uninfected cell CAT activity is given in column 6.



| Plasmid | Relative CAT Activity | | | | Induction |
|----------|-----------------------|------------------|----------|------------------|-----------|
| | Uninfected | Std. Error (+/-) | Infected | Std. Error (+/-) | |
| pLAT1CAT | 1 | 0.11 | 3.62 | 0.61 | 3.62 |
| pLAT7CAT | 0.19 | 0.05 | 2.18 | 0.42 | 11.47 |
| pLAT4CAT | 1.28 | 0.29 | 4.03 | 1.00 | 3.14 |
| pLAT8CAT | 2.79 | 0.27 | 13.35 | 1.95 | 4.7 |
| pLAT6CAT | 5.14 | 1.73 | 47.11 | 19.3 | 9.2 |
| pFJ5 | 0.11 | 0.04 | 0.92 | 0.15 | 8.3 |
| pFJ10 | 48.32 | 8.5 | 612.3 | 89.59 | 12.7 |
| pgDCAT | 4.4 | 0.79 | 534.9 | 94.8 | 121.56 |

Figure 16. The sequence of the LAT6 fragment. The LAT6 sequence was determined from both ends in pT7T3. The first four tracks give the sequence of the opposite (antisense) strand to the sequence displayed on Figure 9. The positions of the various identifiable putative promoter elements are indicated. The position of a C to T transition (G to A on the sense strand) is shown at position -680.



to the promoter activity of pLAT1CAT.

The difference in activity between LAT1 and LAT7 indicates that addition of the 50 bp sequence between the *Hinf*I site at +151 and the *Nae*I site at +201 effects an 8-fold reduction in constitutive activity. A similar result is observed when comparing pIF5 and pLAT5CAT (which have the same 50 bp difference) constitutive activity. The results of pLAT4CAT, pLAT8CAT and pLAT6CAT demonstrate that as the 3' end of the LAT sequence is deleted towards the putative promoter elements, the level of CAT activity increases, with the level of pLAT6CAT activity being greater than that exhibited by pgDCAT (Table 4). This observation is consistent with the notion that these elements form a *bona fide* promoter which exhibits a distance effect, ie, that the measured promoter activity increases as the distance between the promoter and reporter gene decreases.

3.4. Infection Studies

As in section 3C.2, the effect on promoter activity of infecting the transfected cells with HSV-1 was investigated. These results are shown in columns 4 and 5 of Table 4, together with the level of induction above constitutive level of activity indicated in column 6.

The pLAT7CAT result indicates that the 50 bp sequence at its 3' end appears to confer a high amount of relative inducibility (11.5-fold) when compared to pLAT1CAT (3.6-fold), although the actual level of induced pLAT7CAT activity is still less than (60% of) that of pLAT1CAT. This observation reflects the result obtained with pLAT5CAT which also exhibits high inducibility, although to a level greater than that of pLAT7CAT. The infected activity of pLAT4CAT, pLAT8CAT and pLAT6CAT again reflects the importance of the putative promoter elements in directing promoter activity from the LAT sequence. The infected activity increases, again, as the 3' end is deleted towards

the promoter elements, consistent with the distance effect described above. The activity of the strongest LATCAT construct, pLAT6CAT which was induced 9.2-fold, is still, however, less than a tenth of the activity exhibited following infection of cells transfected by either of the lytic cycle promoter constructs (see Table 3 and Figure 4).

3C.4. Deletions from the Centre of the LAT Region

The following section examines the effect on promoter activity of removal of DNA sequences from the centre of the 1141 bp *HinfI/NaeI* LAT region. These experiments were performed at a time before identification of the 8.3 kb LAT, when it was not clear why the promoter was so far upstream from the apparent 5' end of the 2 kb LAT. It was therefore considered that splicing out of a 5' leader sequence between the promoter and 5' end of LAT might be occurring. As in the previous sections, a series of DNA fragments were cloned into pFJ5 and assayed for CAT activity in transfection assays. The location of these fragments are diagrammatically represented in Figure 17; their construction is described below.

4.1. Cloning of the Promoter Sequences

p Δ 1CAT, p Δ 2CAT, p Δ 3CAT: These three plasmids consist of the LAT region between the *HinfI* sites at -940 and +151 with various internal deletions between *NaeI* sites at -549, -341 and -251. These plasmids were generated by partially digesting pLAT1CAT with *NaeI* and religating. Following transformation, colonies were screened and revealed the presence of all three possible permutations resulting from deletions between the *NaeI* sites. Plasmid p Δ 1CAT has a 90 bp deletion between the *NaeI* sites at -341 and -251. Plasmid p Δ 2CAT has a 208 bp deletion between the *NaeI* sites at -549 and -341. Plasmid p Δ 3CAT has a 298 bp deletion between the *NaeI* sites at -549 and -251. The deletions in p Δ 1CAT and p Δ 2CAT remove all of conserved loci 8 and 7

respectively, (see Figure 9) while the deletion in p Δ 3CAT removes both conserved loci.

pLAT4/5CAT: Plasmid pLAT4/5CAT essentially contains the LAT4 and LAT5 sequences fused together to generate a construct similar to LAT7 with a central 144 bp between the *Sma*I site at -395 and *Nae*I site at -251, thus deleting conserved loci 7 and 8. To construct pLAT4/5CAT, the *Hinc*II/*Kpn*I fragment excised from pLAT4 containing the LAT4 sequence was blunt-ended with T4 DNA polymerase and ligated into *Hinc*II-digested pLAT5 DNA to generate plasmid pLAT4/5. The LAT4/5 sequence was excised from pLAT4/5 as a *Sal*I/*Bgl*III fragment and subsequently ligated into *Sal*I/*Bgl*III-digested pFJ5 DNA to generate plasmid pLAT4/5CAT.

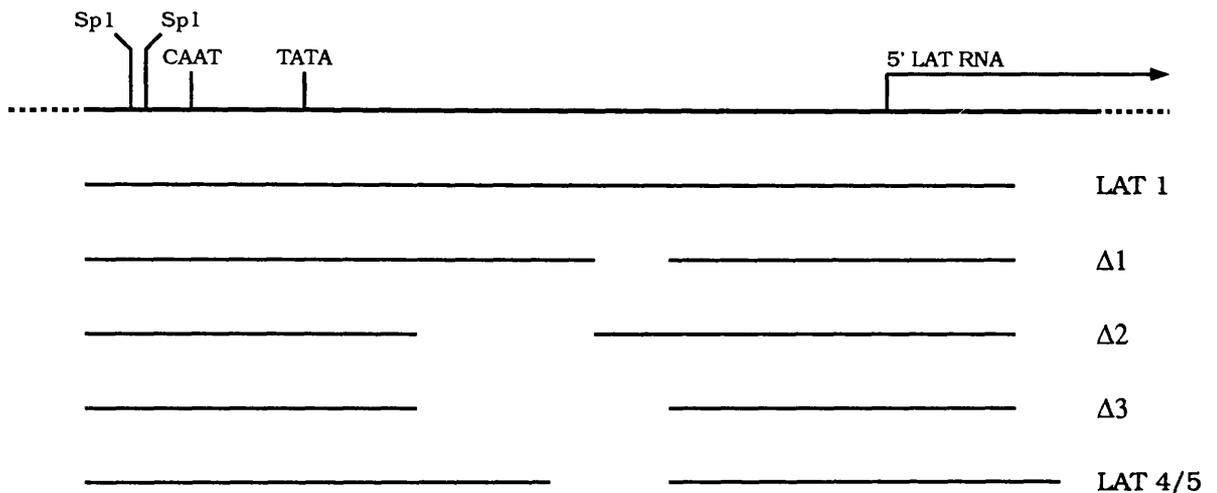
4.2. Expression Assays

The promoter activity of the above constructs were measured by quantitation of CAT activity in short term transfection assays. The results are shown in columns 2 and 3 of Table 5. As before, the data were normalised relative to the promoter activity of pLAT1CAT.

The results indicate that the CAT activity exhibited by p Δ 1CAT is essentially equal to that of pLAT1CAT, thereby demonstrating that the deletion in p Δ 1CAT which removes conserved locus 8 has no effect upon promoter activity exhibited by the LAT sequence. Intriguingly, however, the other constructs, p Δ 2CAT, p Δ 3CAT and pLAT4/5CAT exhibit a relatively high level of constitutive CAT activity, much higher indeed than any of the other LATCAT constructs and pgDCAT. This suggests that the deletions in these constructs have removed some sequence that has a negative effect upon promoter activity. The area of deletion common to all three constructs is a 54 bp sequence between the *Sma*I site at -395 and the *Nae*I site at

Figure 17. Schematic representation of LAT promoter fragments with internal deletions. The top line indicates the relative positions of the putative promoter elements and 5' end of 2 kb LAT. The LAT1 fragment extends from -940 to +151. Δ 1, Δ 2 and Δ 3 are all derived from LAT1 by internal deletions: Δ 1 has a 90 bp deletion from -341 to -261; Δ 2 has a 208 bp deletion from -549 to -341; Δ 3 has a 298 bp deletion from -549 to -251. pLAT4/5CAT is essentially similar to pLAT7CAT with a 144 bp deletion between -395 and -251.

Table 5. Relative CAT activities exhibited by LAT promoter plasmids. The CAT activities exhibited by each of the LAT promoter plasmids were determined and calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1. The mean of at least four independent determinations is given (column 2) with the standard error of the mean (SEM) (column 3). Column 4 records the relative CAT activity exhibited upon infection of transfected cells with 5pfu/cell HSV-1 17+ for 18h, with the SEM given in column 5. The level of induction of CAT activity upon infection, relative to uninfected cell CAT activity is given in column 6.



| Plasmid | Relative CAT Activity | | | | Induction |
|------------|-----------------------|------------------|----------|------------------|-----------|
| | Uninfected | Std. Error (+/-) | Infected | Std. Error (+/-) | |
| pLAT1CAT | 1 | 0.37 | 4.12 | 1.09 | 4.12 |
| Δ1 CAT | 1.14 | 0.38 | 2.6 | 0.86 | 2.27 |
| Δ2 CAT | 18.14 | 7.4 | 11.05 | 2.67 | 0.43 |
| Δ3 CAT | 14.01 | 6.9 | 12.04 | 2.92 | 0.57 |
| pLAT4/5CAT | 7.76 | 3.04 | 95.45 | 29.29 | 12.3 |
| pFJ5 | 0.11 | 0.04 | 0.92 | 0.15 | 8.3 |
| pFJ10 | 48.32 | 8.5 | 612.3 | 89.59 | 12.7 |
| pgDCAT | 4.4 | 0.79 | 534.9 | 94.8 | 121.56 |

-341. Examination of the LAT sequence (Figure 9) shows that this is the area of sequence harbouring conserved locus 7. Since regions of HSV-1/HSV-2 sequence homology are perhaps conserved due to some functional importance, it would be interesting to speculate that conserved locus 7 may function as a repressor of ~~expression~~ .. from the LAT promoter region.

4.3. Infection Studies

The effect on promoter activity of infecting cells transfected with the above constructs was investigated. The results are shown in columns 4 and 5 of Table 5 together with the level of induction indicated in column 6.

The results show that, as in the uninfected cells, the CAT activity exhibited by p Δ 1CAT is essentially similar to that of pLAT1CAT, thus confirming that the deletion of the sequence containing conserved locus 8 has no effect upon promoter activity exhibited by the LAT sequence. Plasmids p Δ 2CAT and p Δ 3CAT exhibit slight reductions of 0.43 and 0.57-fold respectively, thus demonstrating that infection, in contrast to all others, has little effect on the promoter activity exhibited by these constructs. Plasmid pLAT4/5CAT does, however, exhibit an increase of 12.3-fold in CAT activity upon infection. This relatively high level of induction parallels those obtained with pLAT5CAT and pLAT7CAT and again indicates that the 50bp region between the *Hin*FI site at +151 and *Nae*I site at +201 contains some sequence capable of responding strongly to HSV-1 infection.

3C.5. Effect of "upstream" sequences on LAT promoter activity

The following section examines the effect on promoter activity of the addition of sequences upstream of the *Hin*FI site at -940 to the *Dra*I site at -1459, and

therefore containing conserved loci 1 and 2. Two clones were constructed which essentially contained this upstream sequence fused to the LAT1 and LAT6 sequences, and assayed for CAT activity in transfection assays. A schematic representation of these clones is shown on Figure 18.

5.1. Cloning of the Promoter Sequences

pLATOCAT: Plasmid pLATOCAT consists of 1620 bp of LAT sequence encompassing the *DraI* site at -1459 and *HinFI* site at +151, and thus contains all of the recognised putative promoter elements and all of the conserved loci. To construct pLATOCAT, the 638bp *DraI/EcoRV* fragment (corresponding of -1459 to -821 of the LAT sequence) was excised from the *BamHI* *b* containing plasmid pGX48. This fragment was ligated into *HincII/EcoRV* digested pLAT1+*BglIII* to yield plasmid pLAT0. The *HindIII* site in the pUC19 polylinker in pLAT0 was converted to a *Sall* site by *HindIII* digestion and T4 DNA polymerase treatment followed by ligation of the phosphorylated *Sall* linker 5'-GGCGACC-3' to generate plasmid pLAT0-*HindIII+Sall*. The LAT0 sequence was excised from pLAT0-*HindIII+Sall* as a *Sall/BglIII* fragment and subsequently ligated into *Sall/BglIII* digested pFJ5 DNA to generate pLATOCAT.

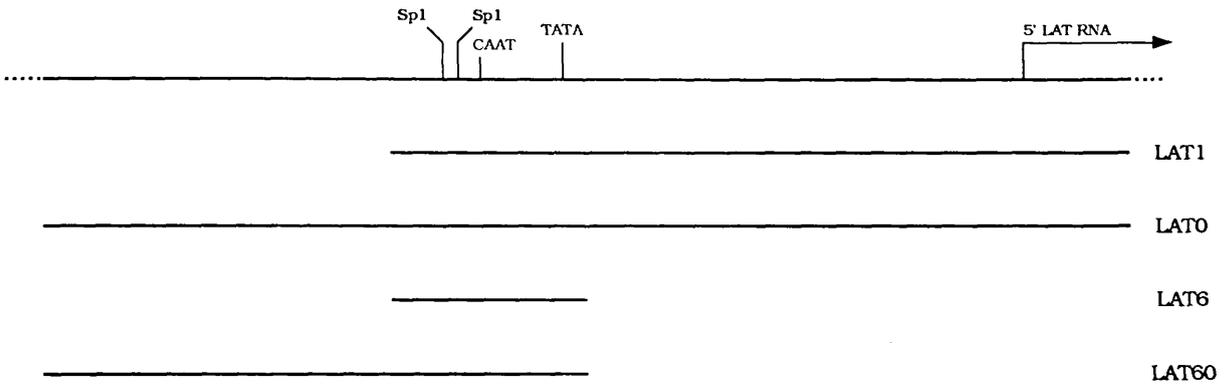
pLAT6OCAT: Plasmid pLAT6OCAT contains 798 bp of LAT sequence spanning the *DraI* site at -1459 and *PvuI* site at -659, and contains all of the putative promoter elements and conserved loci 1 through to 6. To construct pLAT6OCAT, the 661bp *Sall/EcoRV* fragment was excised from pLAT0-*HindIII+Sall* and ligated into *Sall/EcoRV* digested pLAT6 to generate plasmid pLAT60. The LAT60 sequence was excised from pLAT60 as a *Sall/BglIII* fragment and then ligated into *Sall/BglIII* digested pFJ5 DNA to generate pLAT6OCAT.

5.2. Expression Assays

The promoter activity of the above constructs were

Figure 18. Schematic representation of LAT promoter fragments with addition of "upstream" sequences. The top line indicates the relative positions of the putative promoter elements and 5' end of 2 kb LAT. The LAT1 fragment extends from -940 to +151, the LAT0 fragment from -1459 to +151, the LAT6 fragment from -940 to -659, and the LAT60 fragment from -1459 to -659.

Table 6. Relative CAT activities exhibited by LAT promoter plasmids. The CAT activities exhibited by each of the LAT promoter plasmids were determined and calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1. The mean of at least four independent determinations is given (column 2) with the standard error of the mean (SEM) (column 3). Column 4 records the relative CAT activity exhibited upon infection of transfected cells with 5pfu/cell HSV-1 17+ for 18h, with the SEM given in column 5. The level of induction of CAT activity upon infection, relative to uninfected cell CAT activity is given in column 6.



| Plasmid | Relative CAT Activity | | | | Induction |
|-----------|-----------------------|------------------|----------|------------------|-----------|
| | Uninfected | Std. Error (+/-) | Infected | Std. Error (+/-) | |
| pLAT1CAT | 1 | 0.54 | 3.92 | 1.19 | 3.92 |
| pLAT0CAT | 2.74 | 1.19 | 5.98 | 1.34 | 2.42 |
| pLAT6CAT | 11.45 | 2.91 | 136.5 | 32.12 | 11.8 |
| pLAT60CAT | 19.61 | 1.98 | 152.25 | 34.45 | 7.76 |
| pFJ5 | 0.11 | 0.04 | 0.92 | 0.15 | 8.3 |
| pFJ10 | 48.32 | 8.5 | 612.3 | 89.59 | 12.7 |
| pgDCAT | 4.4 | 0.79 | 534.9 | 94.8 | 121.56 |

measured by quantitation of CAT activity in transfection assays. The results are shown in columns 2 and 3 of Table 6. As before, all the data were normalised relative to the promoter activity exhibited by pLAT1CAT.

The results indicate that the effect of addition of upstream sequences to LAT1 and LAT6 in LAT0 and LAT60 respectively is to slightly enhance CAT activity; 2.74-fold in the case of pLAT0CAT and 1.71-fold in the case of pLAT60CAT. This demonstrates that sequence elements upstream of the *HinfI* site at -940, perhaps present in conserved loci 1 and/or 2, form *cis*-acting sequences which function to slightly enhance LAT promoter activity in this assay.

5.3. Infection Assays

The effect on promoter activity of infecting cells transfected with the above plasmids was investigated. The results are shown in columns 4 and 5 of Table 6, together with the level of induction indicated in column 6.

The results show that the level of CAT activity exhibited by pLAT0CAT when infected is 1.5-fold more than that of induced pLAT1CAT, although the actual level of induction is slightly lower (2.4-fold as opposed to 3.92-fold). The level of activity exhibited by pLAT6CAT and pLAT60CAT when infected are similar, although the actual level of induction from constitutive activity is 66% less for pLAT60CAT. This demonstrates that the upstream sequences do not contribute to the level of infected cell CAT activity; all of the sequences responsible for the response to virus infection are therefore confined to the LAT6 fragment.

3C.6. Effect of Vmw110 and Vmw175 on LAT Promoter Activity

The aim of this section is to examine the effect of the HSV-1 immediate-early *trans*-activating polypeptides Vmw110 and Vmw175 upon LAT promoter activity. 3 μ g of the

plasmids pLAT6CAT and pLAT8CAT, chosen since they exhibited high amounts of constitutive CAT activity, and control plasmid pgDCAT were each co-transfected with equal amounts (3 μ g) of p111 (expressing Vmw110), p175 (expressing Vmw175) or both p111 and p175. A total of 9 μ g was used to transfect each BHK monolayer, with pUC19 being used to equalise the amount of DNA in each experiment. The results, normalised for the constitutive activity exhibited by pLAT1CAT are shown on Table 7; standard errors are shown in brackets.

The results show that, as predicted, the gD promoter exhibits a marked response when co-transfected with either p111 or p175, being induced approximately 11-fold and 30-fold respectively. The gD promoter also exhibits the characteristic synergistic effect when co-transfected with both p111 and p175, being induced 138-fold. These data essentially reflects those previously described by Everett, (1984b, 1986).

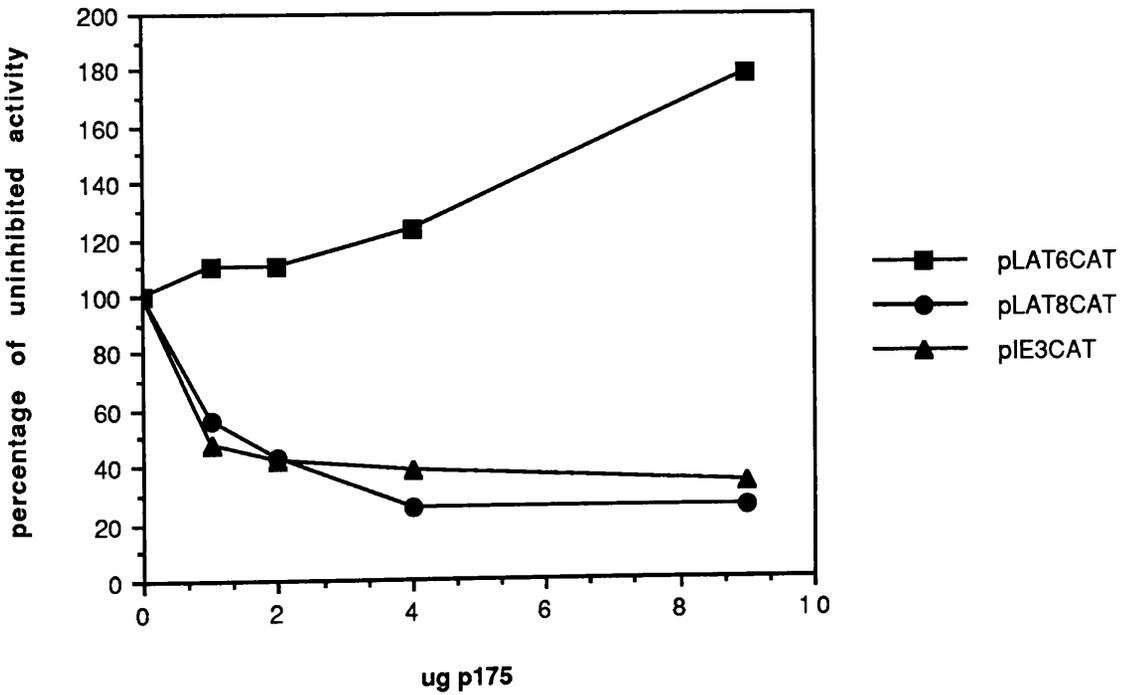
The LAT6 promoter clone exhibits a much weaker response when co-transfected with either p111 or p175 (being induced 1.7-fold and 2.2-fold respectively) with no synergistic effect being observed when co-transfected with both plasmids. The LAT8 promoter fragment exhibits an approximate 5-fold increase in CAT activity when co-transfected with p111. Intriguingly, however, when pLAT8CAT is co-transfected with p175, CAT activity is actually reduced to a level only 78% that of constitutive activity. To further investigate the apparent negative regulatory effect of Vmw175 on CAT expression driven by the LAT8 promoter clone, CAT activity exhibited by pLAT8CAT, pLAT6CAT and control plasmid pIE3CAT was titrated against increasing amounts of p175. The effect of adding increasing amounts of Vmw175-expressing plasmid on the level of IE-3 promoter activity (Paterson & Everett, 1988a), is to increase the level of repression.

Here, 3 μ g of each of pLAT6CAT, pLAT8CAT and pIE3CAT were transfected with increasing amounts (1, 2, 4 and 9 μ g)

Table 7. The effect of Vmw110 and Vmw175 on LAT promoter activity. The CAT activities exhibited by pLAT6CAT, pLAT8CAT and pgDCAT were determined when transfected alone (constitutive activity; column 2), and when co-transfected with Vmw110-expressing plasmid p111 (column 3), Vmw175-expressing plasmid p175 (column 4) or both p111 and p175 (column 5). The mean of 3 independent determinations is given together with the standard error of the mean (shown in brackets). These data were calculated relative to the constitutive activity exhibited by pLAT1CAT (data not shown) which was given the nominal value of 1.

Figure 19. Repression of the LAT promoter by Vmw175. BHK cells were transfected with 3 μ g of either pIE3CAT, pLAT6CAT, or pLAT8CAT, and increasing amounts (1, 2, 4 and 9 μ g) of p175. The total amount of DNA in each transfection was equalised to 12 μ g with pUC19. Cell extracts were prepared 24h after transfection, and their CAT enzyme activities determined, normalised for protein concentration and calculated as a percentage of that obtained in transfections without p175. The symbols representing each plasmid are given in the legend beside the graph.

| Plasmid | Relative CAT Activity | | | |
|----------|-----------------------|------------|------------|-------------|
| | Constitutive | +p111 | +p175 | +p111 +p175 |
| pgDCAT | 3.4 (0.59) | 36.4 (4.4) | 99.6 (7.9) | 496.5 (150) |
| pLAT6CAT | 14.3 (4.8) | 24.2 (5.9) | 31.2 (2.3) | 19.3 (1.5) |
| pLAT8CAT | 4.2 (0.32) | 21.6 (2.9) | 3.3 (0.32) | 7.6 (0.28) |



of p175 in a total of 12 μ g of DNA; pUC19 DNA was again used to equilibrate the amount of DNA in each experiment. The results are represented in graphical form (Figure 19) and calculated as a percentage of CAT activity obtained in transfections without p175. The results show that CAT activity exhibited by pLAT6CAT gradually increases with the amount of p175 to a level almost 80% more than that of uninhibited activity. This increase in activity, albeit small, precludes the possibility of any promoter competition effects between the LAT6 promoter and SV40 early promoter and enhancer driving Vmw175 expression in plasmid p175 (Everett, 1987). The results clearly show that, in accordance with previously published results (Paterson & Everett, 1988a), IE3CAT activity is highly repressed. They also demonstrate that, in contrast to pLAT6CAT, pLAT8CAT promoter activity is reduced in a similar manner to that of the IE-3 promoter. This confirms that LAT8 promoter activity is repressed by Vmw175 *in vitro*, and that this effect must be mediated through the LAT sequence between the PvuI site at -659 and NaeI site at -549.

The ability of Vmw175 to repress transcription from the IE-3 promoter is well documented, (O'Hare & Hayward, 1985) and it has been shown that this effect is mediated through Vmw175 interacting at a specific DNA binding site containing the consensus ATCGTC at the cap site of the IE-3 promoter (Muller, 1987). It was subsequently found that both the integrity of this binding site together with the ability of Vmw175 to bind it were essential for repression (DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, b; Roberts *et al.*, 1988; DiDonato & Muller, 1989). Sequence analysis of the LAT region between the PvuI site and NaeI site reveals that the closest similarity to the core Vmw175-binding consensus is located at -659 to -654 (..ATCGCG..). This homologue occurs at the blunt-ended PvuI site (CGAT'CG) in the LAT6 clone whose 3' terminal residue

is a G immediately preceding the core ATCGTC homologue. A comprehensive sequence comparison is shown on Figure 20 and compares the IE-1 and IE-3 sequences with those of the HSV-1 17+, KOS and HSV-2 HG52^{LAT} sequences. The comparison shows that this sequence, occurring in conserved locus 6 has the preceding three 5' flanking bases and first four bases of the core repression element ie, (..CCGATCG..) of HSV-1 17+, KOS, and HSV-2 HG52 identical to that of the HSV-1 IE-3 sequence. The 3' terminal C residue present in strain KOS increases its homology to the core consensus of the Vmw175 binding site.

It is possible that the occurrence of this potential Vmw175 binding site homology within a region required for Vmw175-mediated repression of expression from the LAT8 promoter is of significance in terms of regulation of LAT gene expression at some crucial point of the virus life cycle.

Figure 20. Vmw175 binding site homology in the LAT promoter. The sequences surrounding the Vmw175 binding sites (ATCGTC) of the HSV-1 IE-3 and IE-1 genes are shown on the top two lines respectively. The Vmw175 homologues in the LAT promoter regions of HSV-1 17+, KOS, and HSV-1 HG52 are shown on the lower three lines respectively. The ATCGTC core homology is shown on the top line.

ATCGTC

GCCCCGATCGTCCACACGGAG
GGGGGAATCGTCACTGCCGCC
GTGCCGATCGCGGGTGGTGCG
GTGCCGATCGCCGGTGGTGCG
AGGCCGATCGAGAGGGACTCC

Core consensus motif

HSV-1 IE-3
HSV-1 IE-1
HSV-1 17 LAT
HSV-1 KOS LAT
HSV-2 HG52 LAT

3D. ANALYSIS OF THE LAT PROMOTER IN A VIRUS VECTOR

3D.1. Introduction

The aim of this study was to examine the ability of the LAT promoter sequences to drive reporter gene expression in the context of the HSV-1 genome during lytic infection of tissue culture cells. The strategy employed was to take LAT promoter/reporter gene fusions selected on their ability to drive gene expression in short term transfection assays (section 3C) and introduce them into the genome of the HSV-1 vector developed by Rixon & McLauchlan, (1990). These recombinant viruses were then used for further characterisation of the LAT promoter constructs during lytic infection of various cell lines.

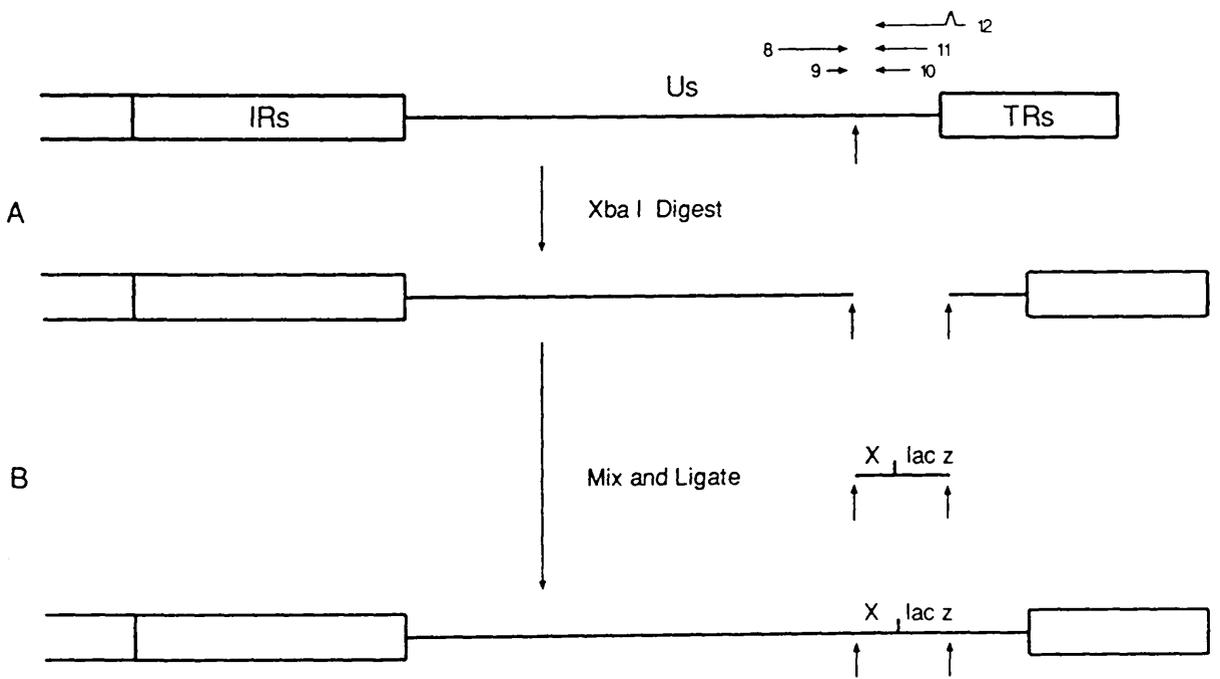
The following sections offer a brief description of the vector, followed by an account of the construction and analysis of the various recombinants employed for the analysis of the LAT promoter.

3D.2. Description of the HSV-1 Vector

The virus vector used in this study employs a system where DNA sequences can be introduced into the HSV-1 genome by direct ligation. This system was developed by the engineering of a single *Xba*I site into an intergenic region of the *Xba*I deficient virus 1702 (MacLean & Brown, 1987b). The *Xba*I site was inserted between *U_s* genes 8/9 and 10/11/12 which are encoded by convergently transcribed genes (Figure 21) and whose 3' termini are separated by some 400 bp of DNA (Rixon & McGeoch, 1985).

A series of plasmids were developed which allows DNA sequences to be placed under the control of various promoters. Each plasmid yields an *Xba*I fragment containing the promoter/gene of interest and the *E.coli lacZ* gene under control of the simian virus 40 (SV40) early promoter. The prototype plasmid used for much of the current work is pFJ5 (Figure 10; Rixon & McLauchlan, 1990) which contains

Figure 21. Strategy for insertion of DNA fragments into HSV-1 1802 DNA. The short region of the HSV-1 genome is shown in the prototype orientation and the *Xba*I sites are indicated by upward-facing arrows. The inverted repeats (IRs and TRs) are represented by open boxes and Us by a single line. The mRNAs (8/9 and 10/11/12) immediately flanking the cloning site are shown. The β -galactosidase gene is represented by *lacZ* and X denotes any additional sequences inserted along with β -galactosidase. This Figure is taken directly from a Figure originally published in Rixon & McLauchlan (1990), with permission.



the chloramphenicol acetyltransferase reporter gene to assay promoter activity. This was also used as the cloning vehicle for the short term transfection assays described in section 3C. The *Xba*I fragment yielded by this plasmid can be ligated into *Xba*I digested vector DNA, designated 1802 (Figure 21), and following transfection, expression of β -galactosidase by recombinant virus in the presence of X-gal results in the production of blue plaques which can be picked and purified.

Rixon & McLauchlan, (1990) placed the CAT reporter gene under the control of HSV IE and early promoters and showed that they were correctly expressed in lytically infected tissue culture cells in both wild-type and *tsk* versions of the vector.

3D.3. Construction of Recombinant Viruses

In the preliminary vector experiments, the *Xba*I fragments from the initial series of LAT plasmids constructed, (pLAT1CAT, pLAT2CAT, pLAT3CAT, pLAT4CAT and pLAT5CAT) were introduced into the 1802 virus vector.

The plasmids were cleaved with *Xba*I and the appropriate fragments isolated and purified. Between 0.5-1 μ g of each fragment was ligated into 0.5 μ g of *Xba*I-digested HSV-1 1802 DNA prior to transfection onto monolayers of BHK cells. Transfections were performed using the calcium phosphate precipitation/DMSO method described by Stow & Wilkie (1976). The progeny virus were harvested and titrated under agar medium. Upon the appearance of virus plaques, typically after 2 days, a further 2ml of agar medium containing 0.75mg/ml X-gal was added to each plate. After incubation overnight, plaques expressing β -galactosidase were picked. All viruses were purified to homogeneity typically through three to four rounds of plaque purification. All viruses were designated "vLC" eg, the virus derived from plasmid pLAT1CAT is termed vLC1.

3D.4. CAT Expression from Recombinant Viruses

The levels of CAT activity produced by the LATCAT fusion viruses along with controls vFJ5, vFJ7 and vFJ10 were determined at 6, 12, 24 and 48 hours after infection with a moi of 5 at 37°C. The controls vFJ7 and vFJ10 have the HSV-2 ribonucleotide reductase small subunit (early gene) and HSV-2 IE-4/5 promoter (immediate-early gene) promoters driving CAT activity respectively, while vFJ5 has no promoter to drive CAT activity. A typical set of data is shown on Figure 22. For sake of clarity the data is presented as a whole on a logarithmic plot (Figure 22(a)) while the data for the vLC viruses alone are presented on a linear plot (Figure 22(b)).

Figure 22(a) shows that vFJ7 and vFJ10 exhibit essentially similar patterns of CAT activity in agreement with Rixon & Mclauchlan (1990). Their activity is 2-3 logs higher than that exhibited by any of the vLC viruses. This result parallels those obtained with the infected transfection assays (see Tables 3 and 4) where CAT activities exhibited by pFJ10 and pgDCAT were similarly between 2 and 3 logs greater than those obtained with any of the corresponding LATCAT plasmids. The vFJ5 control virus exhibits negligible activity, thereby confirming that the activity exhibited by infected pFJ5 (Table 3) is a consequence of cryptic promoters within the pFJ5 vector sequence. Figure 22(b) shows that the activities exhibited by the vLC viruses do not reach appreciable levels until 24h pi, whereas the lytic promoters reach a plateau of activity by 12h pi. vLC1 yields a level of CAT activity, which, as similarly observed in the transfection assays (Table 3), is reduced by two-thirds in vLC2, and further reduced in vLC3. This confirms that the sequences capable of functioning as a promoter in the context of the virus genome are similar to those defined by the transfection assays. While vLC4 and vLC5 exhibit rather less activity

relative to vLC1 than their counterparts in the transfection assays, vLC5 still exhibits a relatively high activity, thus confirming the results of the infected transfection assays (Table 3), that the LAT5 clone contains sequences that can function as a promoter in the context of viral infection.

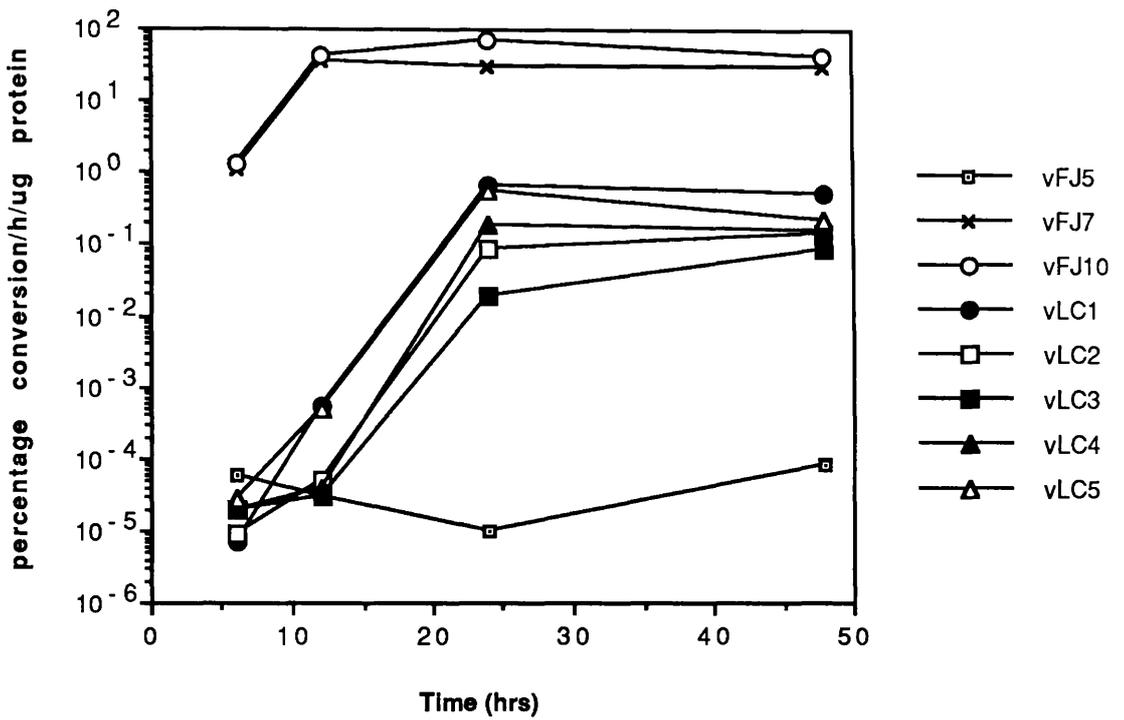
These results demonstrate that the LAT constructs behave in a similar fashion in the context of the virus genome, thus maintaining the same general conclusions arrived at from the transfection assays, ie, that the sequences responsible for promoter activity within the LAT sequence are within the region harbouring the recognisable putative promoter elements.

3D.5. Analysis of Virus Genomes

The introduction of a third copy of the LAT sequences into the virus vector genome raised the possibility of genome rearrangements occurring as a result of homologous recombination, thus making the interpretation of any results obtained with these viruses liable to substantial error. To determine the genome structure of the 1802-based LAT promoter/CAT fusion viruses, each purified virus was used to infect 35mm dishes of BHK cells. When complete cpe was observed, total DNA was extracted and analysed by restriction digestion with *HindIII* and *XbaI*, run on a 1% agarose gel alongside the corresponding *HindIII/XbaI* digested LATCAT plasmids, followed by Southern transfer to nitrocellulose filters and probing with the nick translated *XbaI/HindIII* LAT promoter fragment derived from pLAT1CAT (Section 3C.2.1). Figure 23 shows that all viral DNAs yield LAT promoter fragments of similar size to those from their corresponding plasmids. Perhaps suprisingly, there are no obvious signs of viral genome rearrangements, thereby demonstrating that CAT activity derived from these viruses gives a true reflection of the relative promoter activities exhibited by the various LAT

Figure 22. CAT activities produced by recombinant 1802 viruses at various times after infection. a). CAT activities generated by vFJ5, vFJ7, vFJ10 and LAT promoter viruses vLC1 to vLC5 shown on a logarithmic scale. **b)** CAT activities generated by the LAT promoter viruses alone, shown on a linear scale. CAT activities were determined at 6, 12, 24 and 48 h following infection and expressed as the percentage [¹⁴C]chloramphenicol converted per h per μg protein. The symbols representing each virus are given in the legends beside each graph.

a)



b)

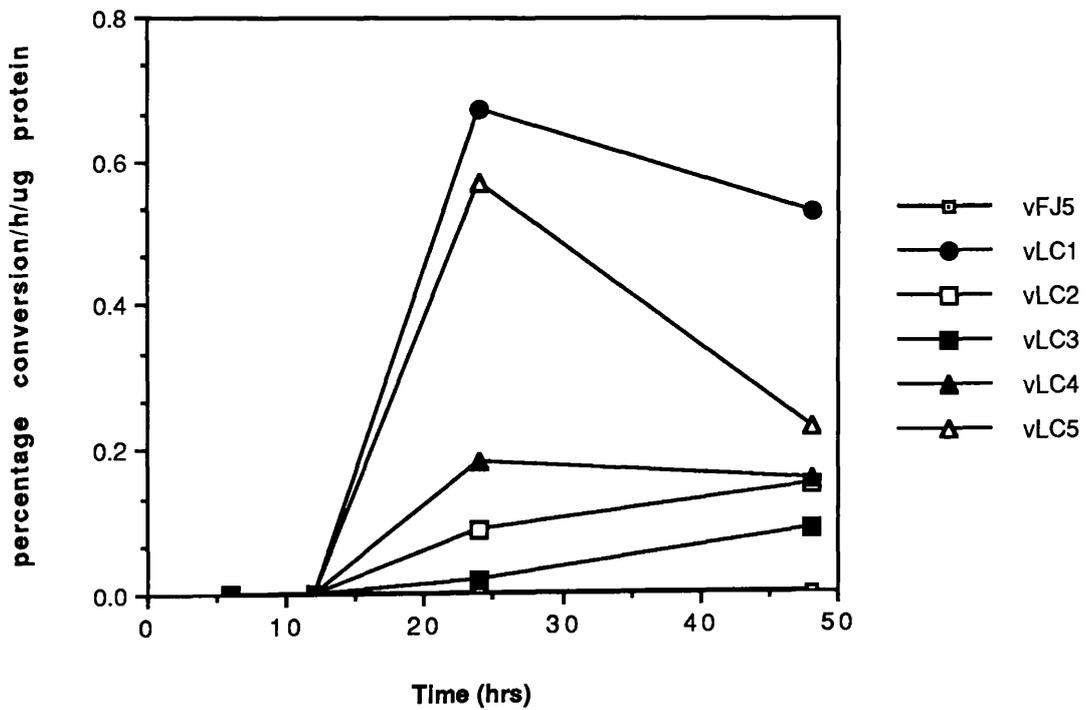


Figure 23. Southern blot analysis of the 1802-derived LAT promoter/CAT fusion viruses. Viral DNA was prepared from infected cells when extensive cpe was apparent. Each viral and corresponding plasmid DNA was digested with *Xba*I and *Hind*III and subjected to Southern blot analysis using a nick translated *Xba*I/*Hind*III LAT promoter probe derived from pLAT1CAT.

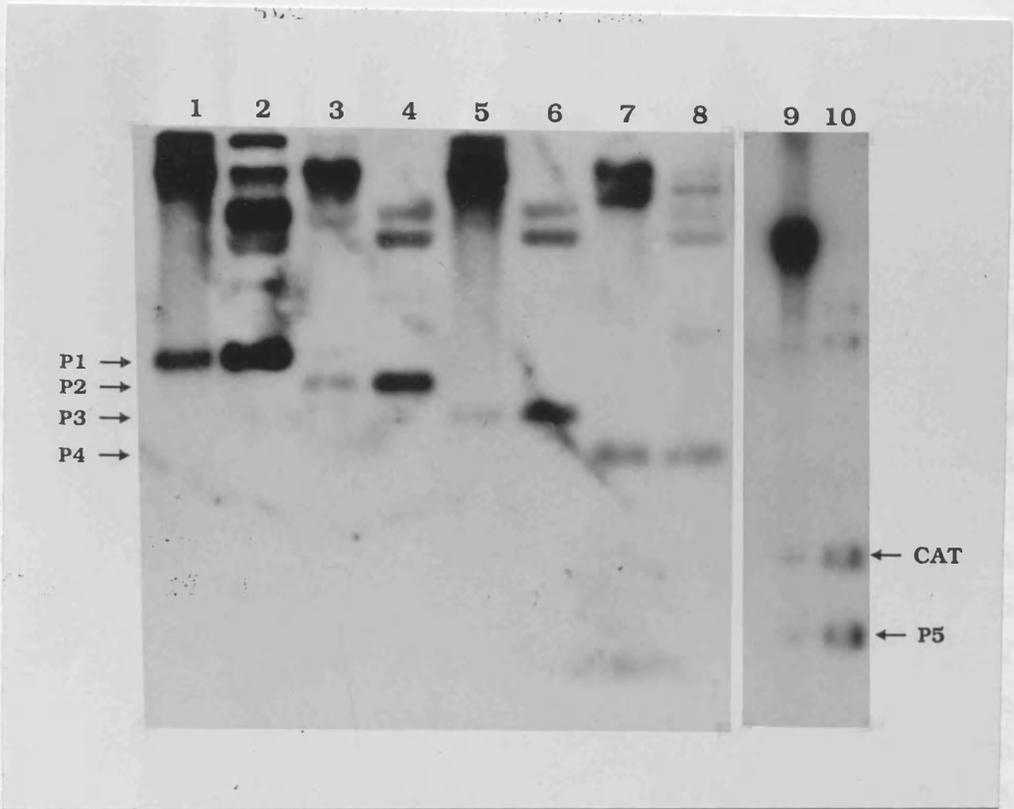
| | Band size: (bp) |
|---------------------------------|-----------------|
| Track 1: vLC1 infected cell DNA | |
| Track 2: pLAT1CAT | 1091 |
| Track 3: vLC2 infected cell DNA | |
| Track 4: pLAT2CAT | 949 |
| Track 5: vLC3 infected cell DNA | |
| Track 6: pLAT3CAT | 746 |
| Track 7: vLC4 infected cell DNA | |
| Track 8: pLAT4CAT | 545 |
| Track 9: vLC5 infected cell DNA | |
| Track 10: pLAT5CAT | 452 |

The positions of the LAT promoter fragments from each recombinant virus are indicated (from P1 to P5). The strongly hybridizing bands at the top of each virus infected cell DNA track is due to hybridization of the LAT promoter probe to the endogenous HSV-1 LAT sequences. The CAT band shows up in lanes 9 and 10 due to contamination of the probe with CAT sequences from pLAT1CAT in that particular experiment.

protein/CAT fusion in the context of each recombinant virus genome.

2.2. CONSTRUCTION OF A CAT-DRIVEN VERSION OF BVDV-1002

2.2.1. Strategy



protein activity. This is a primary objective of the present study. A representative representation of the strategy used for the construction and cloning of the CAT-driven BVDV-1002 is shown in Figure 2.

2.2.2. Construction of BVDV-1002

As *Bam*HI is a rare restriction enzyme and a straightforward *Bam*HI/*Ap*RI strategy was not feasible, instead, *Bam*HI is a 1000 bp fragment was excised from pUC19 as a *Bam*HI fragment and ligated into the *Bam*HI site of the pUC19 polyliner whose additional restriction sites were further manipulated prior to perform the above

promoter/CAT fusions in the context of each recombinant virus genome.

3E. CONSTRUCTION OF A LAT-DELETED VERSION OF HSV-1 1802

3E.1. Strategy

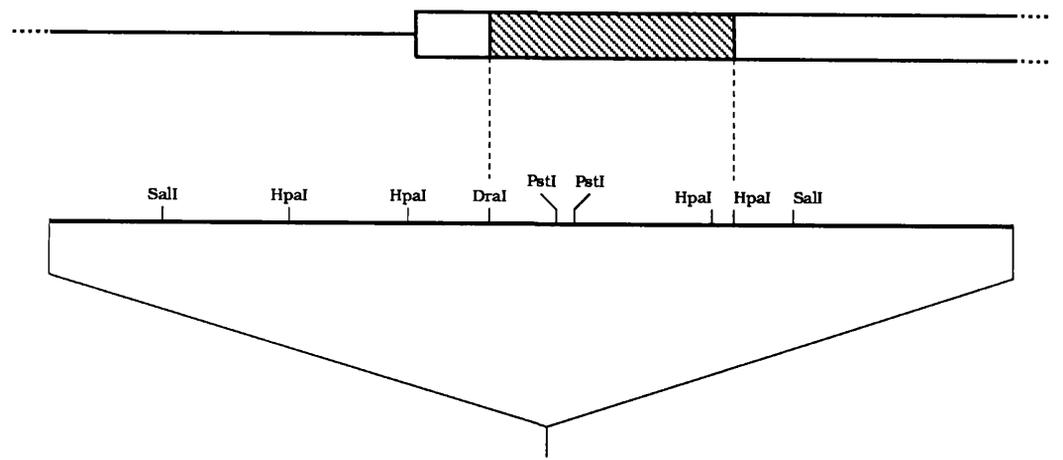
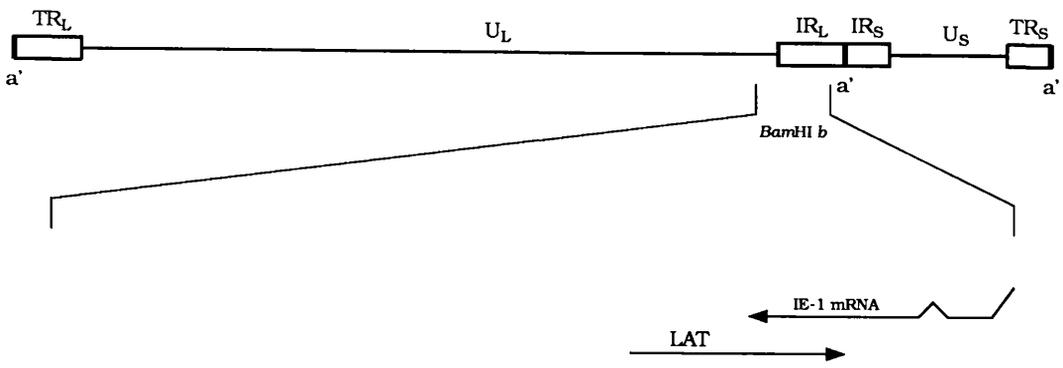
Although the genome structures of the above viruses appeared normal, it was nevertheless decided to construct a version of the vector in which the endogenous LAT sequences are deleted, thus allowing a more confident interpretation of any results obtained when characterising viruses containing LAT promoter/ CAT fusion sequences.

The strategy involved deleting a proportion of the LAT sequences from HSV-1 *Bam*HI *b* and *e* of the virus genome by co-transfection of LAT-deleted *Bam*HI *b* DNA with 1802 DNA and screening progeny plaques for virus harbouring deletions in both copies of the LAT gene. The sequence deleted from *Bam*HI *b* was a 2466bp region spanning from the *Dra*I site (HSV-1 sequence position 118002; also the 5' terminus of the LAT0 and LAT60 clones) to a *Hpa*I site (HSV-1 sequence position 120468) 370bp downstream of the 3' end of the IE-gene 1 mRNA, thereby deleting all of the endogenous HSV-1 sequences under examination for LAT promoter activity. Figure 24 presents a diagrammatic representation of the deletion from the HSV-1 genome and summarises the construction of the LAT-deleted *Bam*HI *b* plasmid.

3E.2. Construction of pBdelLAT

As *Bam*HI *b* contains three additional *Hpa*I sites, a straightforward *Dra*I/*Hpa*I digestion and religation was not feasible. Instead, *Bam*HI *b* in pAT153 was excised from pGX48 as a *Bam*HI fragment and ligated into the *Bam*HI site of the pUC19 polylinker whose additional restriction sites made further manipulations easier to perform. The chosen

Figure 24. Construction of LAT-deleted BamHI b. The top line depicts the HSV-1 genome. The location of *Bam*HI *b* is shown and expanded on the line below. The U_L region is shown as a single line and IR_L as a box. The relative positions of the 2kb LAT and IE-1 mRNA are shown above the box. The shaded region within IR_L represents the *Dra*I/*Hpa*I region to be deleted. A partial restriction map of *Bam*HI *b* is shown on the next line below. *Bam*HI *b* was excised from plasmid pGX48 as a *Bam*HI fragment and ligated into the the *Bam*HI site of pUC19 in the orientation indicated. As shown in the lower right hand side of the Figure, this plasmid (pBamB) was *Pst*I digested and ligated, thus removing sequences between the *Pst*I site in the pUC19 polylinker and proximal *Pst*I in *Bam*HI *b*, thereby generating plasmid pBdelPst. This plasmid was digested with *Hind*III and *Hpa*I, thereby removing the sequences between the *Hind*III sites in the pUC19 polylinker and 3' *Hpa*I site in *Bam*HI *b*. This vector was mixed and ligated with a *Dra*I/*Hind*III fragment purified from pBamB (see lower left hand side of the Figure) to generate plasmid pBdelLAT. A partial restriction enzyme map of pBdelLAT is shown on the bottom line.



---- HindIII SphI PstI SalI XbaI BamHI SmaI KpnI SstI EcoRI ---- pUC19 polylinker

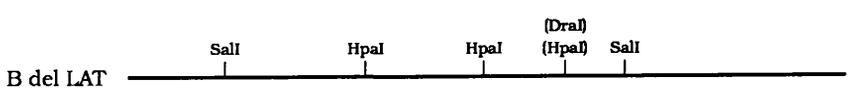
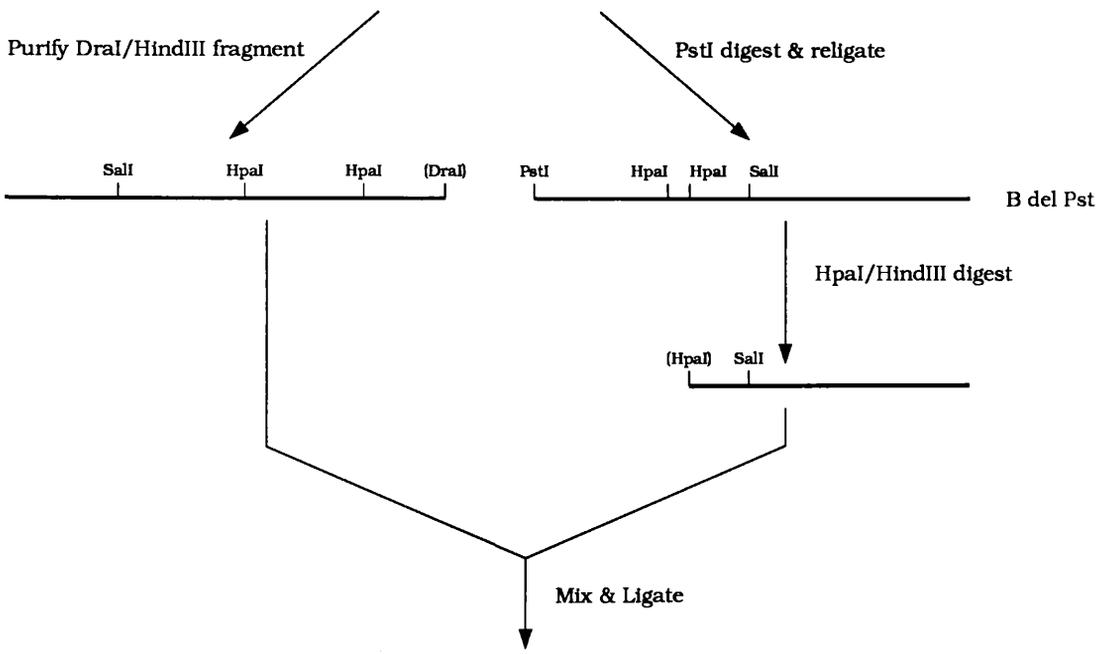


Figure 25. Restriction enzyme analysis of LAT-deleted *Bam*HI *b* plasmids. Plasmids pBamB, pBdel Pst and pBdelLAT were digested with *Sal*I and run on a 1% agarose gel along with 1 kb size markers (lane 4). Plasmid pBdelPst (lane 2) has a single *Sal*I site, and as expected, runs as a single band of 7198 bp. The parental plasmid pBamB (lane 1) which has the complete *Bam*HI *b* sequence runs as 3 bands of 6283, 5248 and 1196 bp. The LAT-deleted pBdelLAT (lane 3) has a 2466 bp deletion from the 6283 bp fragment thus giving rise to a 3817 bp band on the gel.

orientation of *Bam*HI sites in pBC19 is shown in Figure 24. This plasmid (pBC19) was digested with *Bam*HI and religated, thereby deleting all sequences between the *Bam*HI site in the pBC19 polylinker and the *Bam*HI site in *Bam*HI b. The resulting plasmid, pBC19b, was digested with *Hind*III and *Sma*I, then ligated with *Sma*I purified *Hind*III/*Dra*I fragment from pBC19b to generate pBC19bAT. Diagnostic restriction enzyme digests of pBC19b and pBC19bAT are shown on Figure 25.

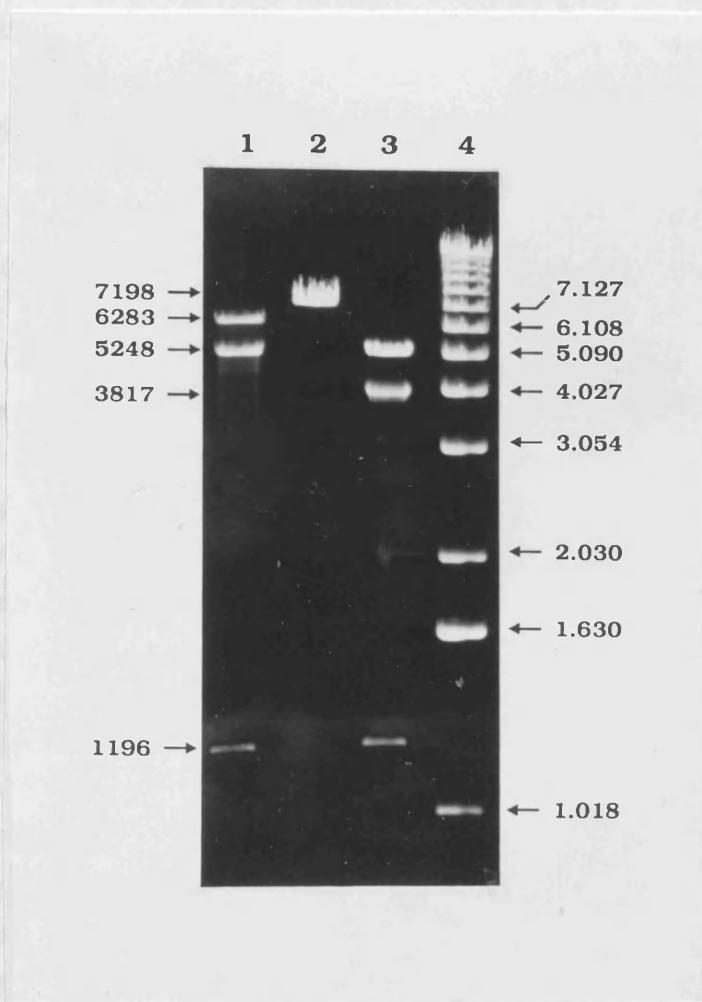
32.3. Construction

0.5µg of co-transformed was plated on 25cm plates of BHK cells from each plate into 16 groups 50cm dishes from recombinant plasmids.

SalI digests, Southern blotting and probe fragment isolation. The location of the recombinant is virus yields. A repeat unit, 49 deletions from fragments of 633 on the Southern relatively short

derived probe and the SalI site and various other

Using this strategy, 1000 plaques of plaques, nos. 1(7-13), 2(1-13) and 3(1-13) were positive (Figure 26). The remaining 1000 plaques were then further tested, and 1000 were positive (11/9, 15/3, 5/8, 11/12 and 10/13). These plaques



orientation of *Bam*HI *b* in pUC19 is shown in Figure 24. This plasmid (pBamHI*b*) was digested with *Pst*I and religated, thereby deleting all sequences between the *Pst*I site in the pUC19 polylinker and the distal *Pst*I site in *Bam*HI *b*. The resulting plasmid, (pBdel*Pst*), was digested with *Hind*III and *Hpa*I, then ligated with the purified *Hind*III/*Dra*I fragment from pBamHI*b* to generate pBdelLAT. Diagnostic restriction enzyme digests of pBam*B*, pBdel*Pst* and pBdelLAT are shown on Figure 25.

3E.3. Construction of the LAT-Deleted HSV-1 Vector

0.5µg of plasmid pBdelLAT (digested with *Bam*HI) was co-transfected with 0.5µg of HSV-1 1802 DNA onto eight 50mm plates of BHK monolayers. A total of 96 progeny plaques (12 from each plate) were picked, and a quarter of each pooled into 16 groups of 6. Each group was then used to infect 50mm dishes from which DNA was prepared and analysed for recombinant plaques.

*Sal*I digested viral DNA was analysed by Southern blotting and probing with the nick-translated 6283bp *Sal*I fragment isolated and purified from *Bam*HI *b*. The genomic location of the probe used for screening the desired recombinant is illustrated on Figure 26. The parental 1802 virus yields 2 bands of 8768 and 6385 bp (one from each repeat unit; see Figure 24), while virus with the 2466bp deletions from *Bam*HI *e* and *Bam*HI *b* gives rise to *Sal*I fragments of 6302 and 3919 bp respectively. The upper bands on the Southern blot will appear fainter due to the relatively short area of homology between the *Bam*HI *b*-derived probe and the homologous region between the *Sal*I site and terminus of TR_L in *Bam*HI *e*.

Using this diagnostic test, four groups of plaques, nos. 1(7-12), 5(1-6), 7(7-12) and 8(1-6) were positive (Figure 26). The original plaques from each group were then further tested, and from these, five plaques were positive (1/9, 5/3, 5/5, 7/11 and 8/5; see Figure 27). These plaques

were titrated out, and a further five progeny plaques picked, termed 1/9(A to E) etc. As indicated on Figure 28, all 1/9 plaques, 5/5A, 5/5D and 8/5C look as if they carried the deletion. Examination of the 1/9 genome by ^{32}P DNA labelling revealed that 1/9 had gained an extra *Xba*I site. A further 10 plaques from 8/5C were therefore picked and DNA made from 35mm plates. Nos. 5, 6, 7 and 9 (Figure 29) all carried the deletion and had been purified to homogeneity. One of these was chosen and designated 1804. This LAT⁻ virus was used for all subsequent analysis of the LAT promoter constructs.

3F. Analysis of the LAT Promoter in the 1804 Vector

3F.1. Introduction

In the following series of experiments, it was decided to characterise the LAT constructs centred around the LAT promoter as defined by the transfection experiments. To this end, the *Xba*I fragments from plasmids pLAT1CAT, pLAT4CAT, pLAT8CAT, pLAT6CAT and pLAT60CAT were isolated, purified and introduced into the 1804 vector as described in section 3D.3. All of these 1804-derived viruses were designated with the prefix "v", such that, for example, the virus derived from pLAT1CAT is designated vLAT1CAT. These viruses were subsequently used for further characterisation of the LAT promoter during lytic infection of BHK and C1300 neuroblastoma cell lines.

3F.2. Analysis of Viral Genomes

Total DNA was prepared from BHK monolayers prepared with each purified virus and analysed by restriction enzyme digestion with *Hind*III/*Xba*I, running on a 1% agarose gel alongside the corresponding *Hind*III/*Xba*I digested LATCAT plasmids, followed by Southern transfer to nitrocellulose filters and probing with the nick translated *Xba*I/*Hind*III LAT promoter fragment derived from pLAT1CAT (Section 3C.2.1). Figure 30 shows that all LATCAT viral DNAs yield

Figure 26. Screening for LAT-deleted virus. A schematic representation of the long region of the HSV-1 genome, together with specified restriction enzyme sites, are shown on the top line. The shaded areas in TR_L and IR_L represent the *DraI/HpaI* regions to be deleted. The region to be deleted from TR_L lies in *BamHI e*, and that from IR_L from *BamHI b*. Recombinant virus genomes were screened by *SalI* digestion and probing with a *SalI* probe derived from *BamHI b*. The parental 1802 virus should give rise to *SalI* fragments of 8768 and 6385 bp, while viruses carrying 2466 bp deletions from both *BamHI b* and *BamHI e* should give rise to *SalI* fragments of 6302 and 3919 bp. Since the 6385 and 6302 bp fragments co-migrate with each other on the gels, recombinant viruses were initially screened for the presence of the 3919 bp band.

The blots below show the screening of the original 96 picked plaques (12 from each of the original plates transfected). Fractions of each group of twelve were pooled into 2 groups of 6. 'A' represents pooled plaques nos. 1 to 6, and 'B', nos. 7 to 12. This gave a total of 16 pooled groups. The digests of viral DNA were compared with similar digests of pBamB (in the lane marked BamB) and pBdelLAT (in the lane marked Bdel). The sizes of the various bands are given on the right hand side of each blot. The 6302 and 6385 bp bands are indistinguishable on these blots. The band marked 'vector' is the pUC19 backbone of the plasmid from which the probe was excised before radioactive-labelling. This band shows up on this blot due to contamination with vector sequences during purification of the probe.

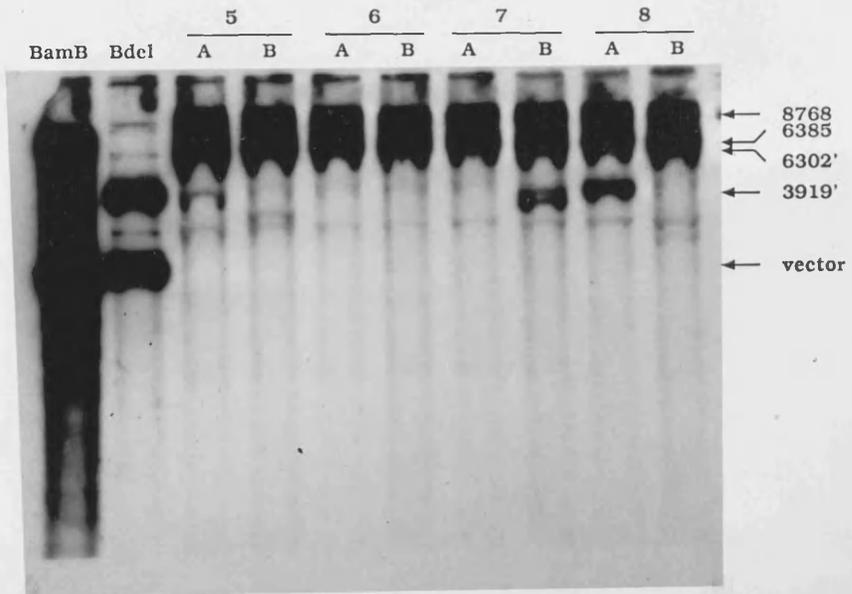
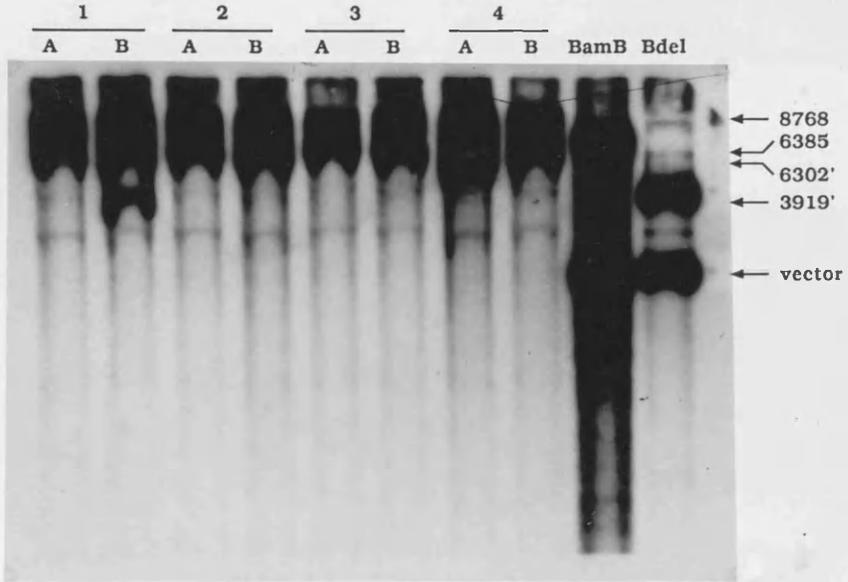
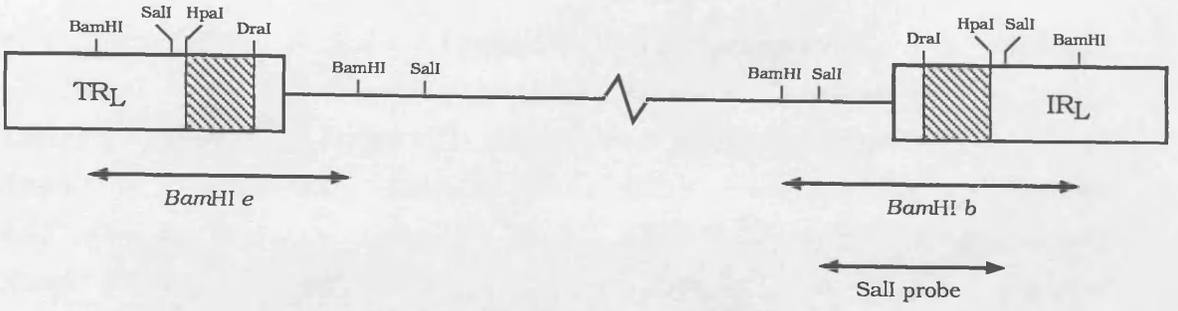


Figure 27. Screening of original picked plaques. The original plaques from each pooled group which appeared to harbour the appropriate deletions (Figure 26) were further tested, and compared with *Sal*I digests of plasmids pBamB (lane BamB), and pBdelLAT (lane Bdel). The sizes of the various bands are given on the right hand side of each blot. This analysis indicated that five plaques (1/9, 5/3, 5/5, 7/11, and 8/5) were positive.

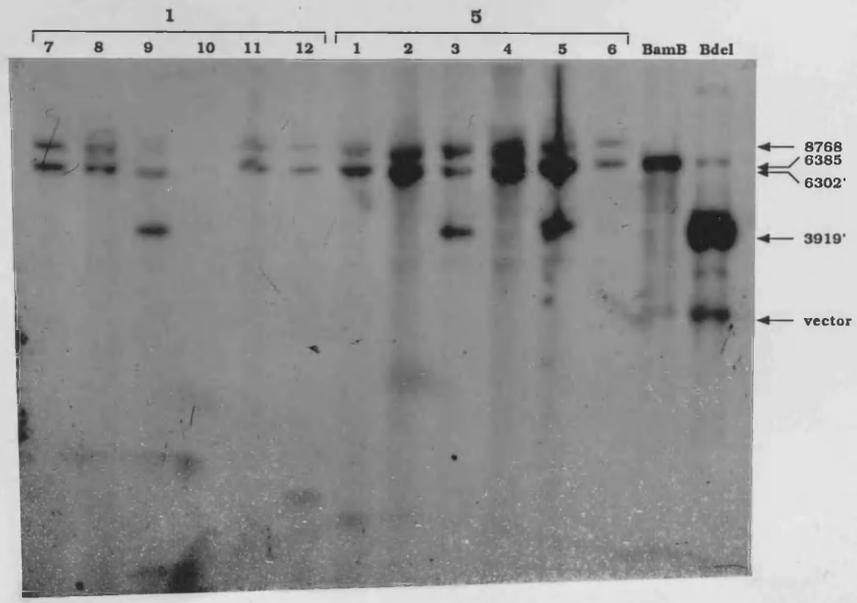
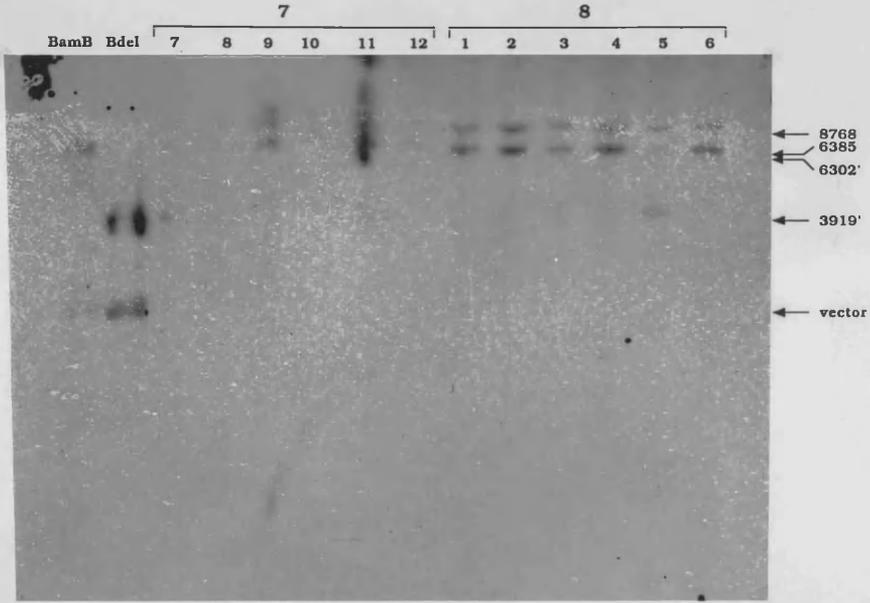


Figure 28. Sreening of second round of picked plaques. The positive plaques from the first round of plaque-picking (Figure 27) were titrated out, and a further five progeny plaques (termed A to E) picked. These were again compared to similar digests of pBamB (lane BamB) and pBdelLAT (lane Bdel). The sizes of the relewant bands are again given beside each blot. This analysis demonstrated that all 1/9 plaques, 5/5A, 5/5D and 8/5C were positive.

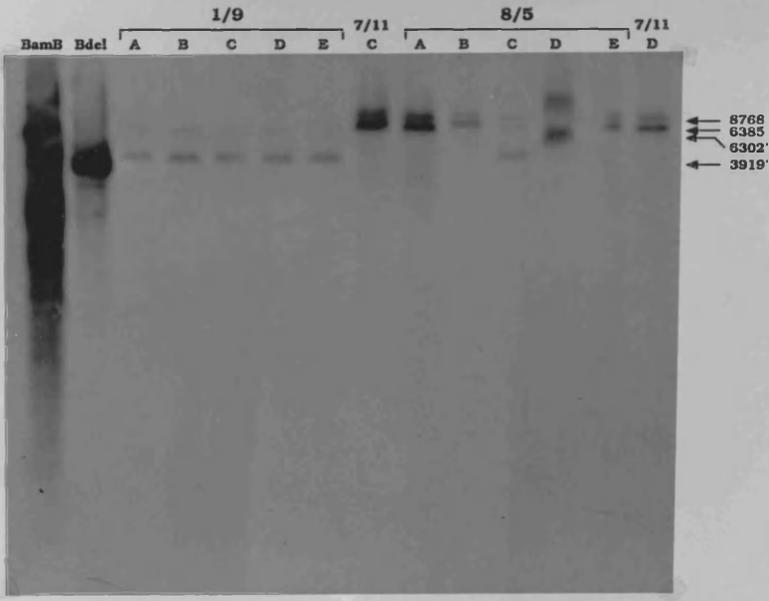
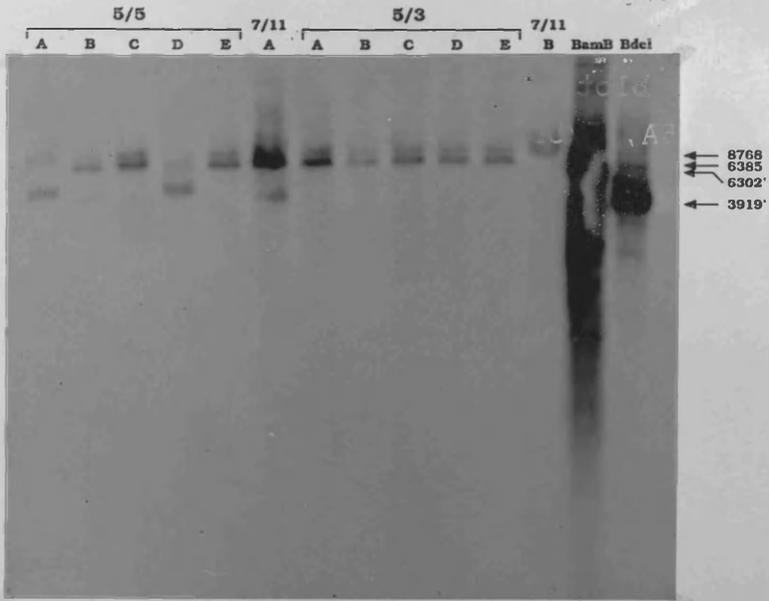


Figure 29. Titration of 8/5C. 8/5C (Figure 28) was titrated, and a further 10 plaques (designated 1 to 10) picked and tested. These were again compared to similar digests of pBamB (lane BamB) and pBdelLAT (lane Bdel). The sizes of the relevant bands are again given beside the blot. This analysis demonstrated that nos. 5, 6, 7 and 9 were pure.

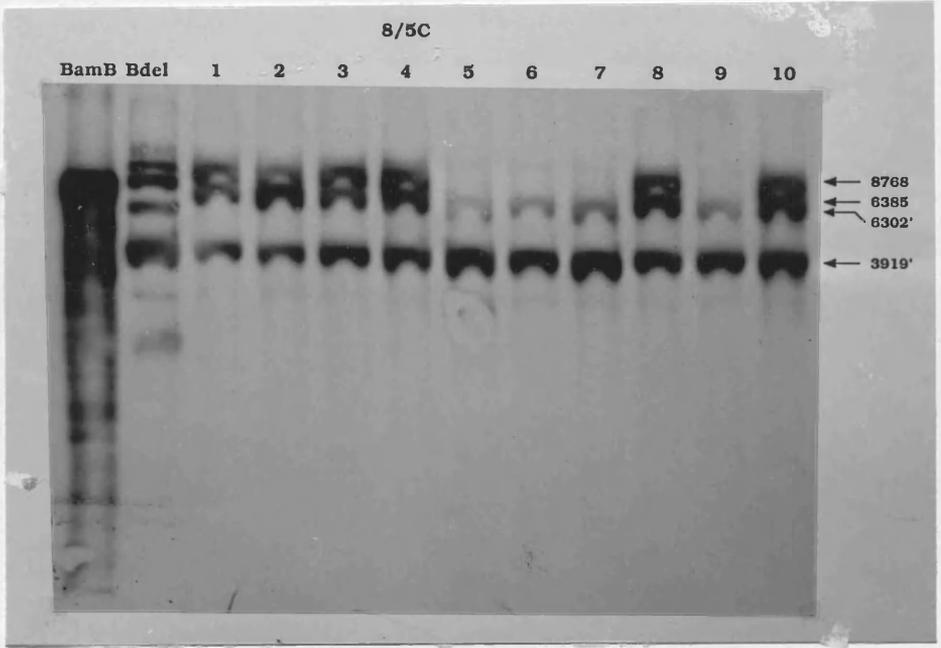


Figure 30. Southern blot analysis of DNA prepared from 1804-derived virus infected cells. Viral DNA was prepared from infected cells when extensive cpe became apparent. Each viral and corresponding plasmid DNA was digested with *Xba*I and *Hind*III and subjected to Southern blot analysis using a nick translated *Sal*I/*Eco*RI LAT promoter probe (containing all of the LAT promoter sequences) derived from pLAT1 (see Section 3C.2.1).

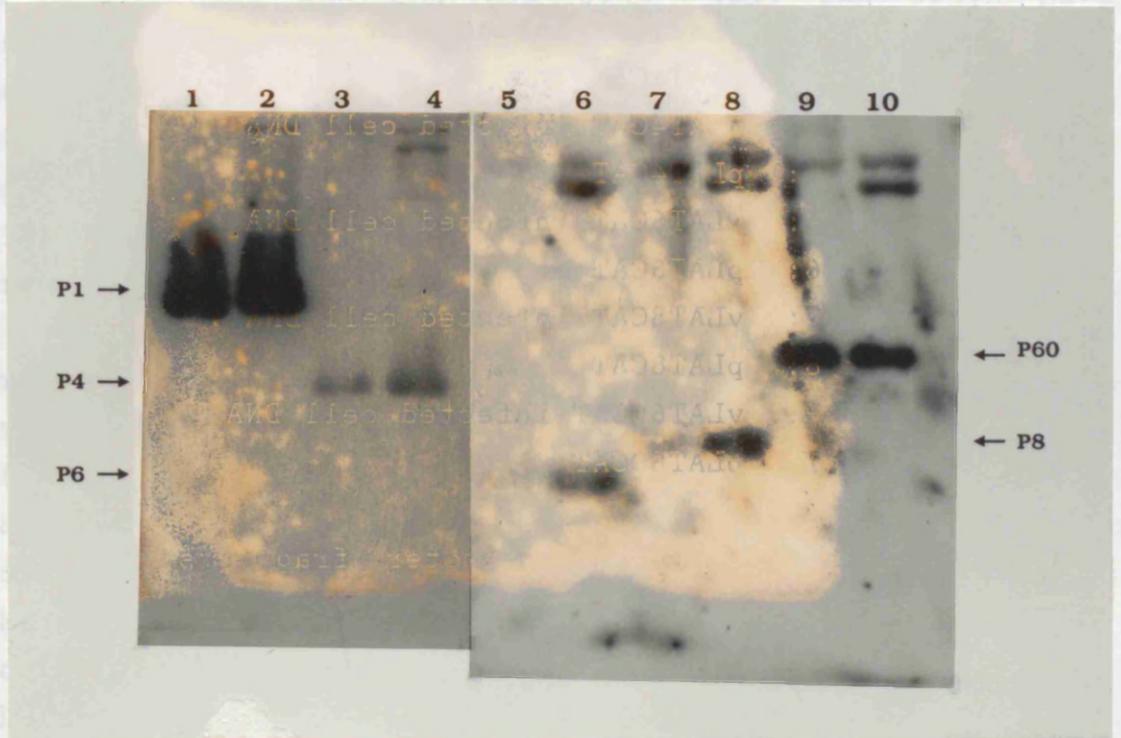
| | Band Size (bp) |
|--------------------------------------|----------------|
| Track 1: vLAT1CAT infected cell DNA | 1099 |
| Track 2: pLAT1CAT | |
| Track 3: vLAT4CAT infected cell DNA | 949 |
| Track 4: pLAT4CAT | |
| Track 5: vLAT6CAT infected cell DNA | 746 |
| Track 6: pLAT6CAT | |
| Track 7: vLAT8CAT infected cell DNA | 545 |
| Track 8: pLAT8CAT | |
| Track 9: vLAT60CAT infected cell DNA | 452 |
| Track 10: pLAT60CAT | |

The positions of the LAT promoter fragments from each recombinant virus are indicated (from P1 to P60). Note that the lack of extensive hybridization at the top of each viral DNA track (compared with Figure 23) is further confirmation that the 1804-derived viruses are deleted for the endogenous LAT sequences.

LAT promoter fragments of similar size to those from their corresponding plasmids. The LAT promoter probe also, as expected, fails to detect any of the analogous LAT sequences which have been deleted from the 1000 vector. For

3.3. CAT Expression from Recombinant Viruses

3.1. BHK Cells. The levels of CAT activity produced by the LAT1000 virus and controls p336, p337, and p338 were



viAT1CAT, and that this activity is increased as the distance between the LAT promoter and CAT reporter gene is reduced in the LAT1000 fusions as observed with viAT5CAT and viAT6CAT. viAT6CAT exhibits slightly less activity than viAT5CAT, thereby confirming the transcriptional repression (Table 1), demonstrating that sequences upstream of the LAT6 clone are not necessary for LAT promoter activity in BHK cells.

In conclusion, these results demonstrate that the LAT promoter constructs behave in a similar manner in the context of the HSV-1 genome as they do in the transcription assays, and that the sequences upstream and necessary for LAT promoter expression during lytic infection of tissue

LAT promoter fragments of similar size to those from their corresponding plasmids. The LAT promoter probe also, as expected, fails to detect any of the endogenous LAT sequences which have been deleted from the 1804 vector.

3F.3. CAT Expression from Recombinant Viruses

3.1. BHK Cells. The levels of CAT activity produced by the LATCAT viruses and controls vFJ5, vFJ7 and vFJ10 were determined at 6, 12, 24 and 48 hours post infection at a moi of 5 at 37°C. A typical set of data is shown of Figure 31. As in section 3D.4, for sake of clarity, the data is again presented as a whole on a logarithmic plot (Figure 31(a)) while the data from the LATCAT viruses alone are presented on a linear plot (Figure 31(b)).

Figure 31(a) shows that the CAT activities exhibited by the lytic cycle promoters in vFJ7 and vFJ10 are at least a log greater than that exhibited by the strongest of the LATCAT viruses (vLAT6CAT). This, as with the 1802 vector results, parallels those obtained with the corresponding plasmids in the transfection experiments. This pattern is maintained when examining the relative CAT activities of the LATCAT viruses. Figure 31(b) shows that vLAT4CAT exhibits increased levels of activity over vLAT1CAT, and that this activity is increased as the distance between the LAT promoter and CAT reporter gene is reduced in the LATCAT fusions as observed with vLAT8CAT and vLAT6CAT. vLAT60CAT exhibits slightly less activity than vLAT6CAT, thereby confirming the transfection results (Table 6), demonstrating that sequences upstream of the LAT6 clone are not necessary for LAT promoter activity in BHK cells.

In conclusion, these results demonstrate that the LAT promoter constructs behave in a similar manner in the context of the HSV-1 genome as they do in the transfection assays, and that the sequences sufficient and necessary for LAT promoter expression during lytic infection of tissue

culture cells lie within the 277bp *HinfI/PvuI* LAT6 sequence.

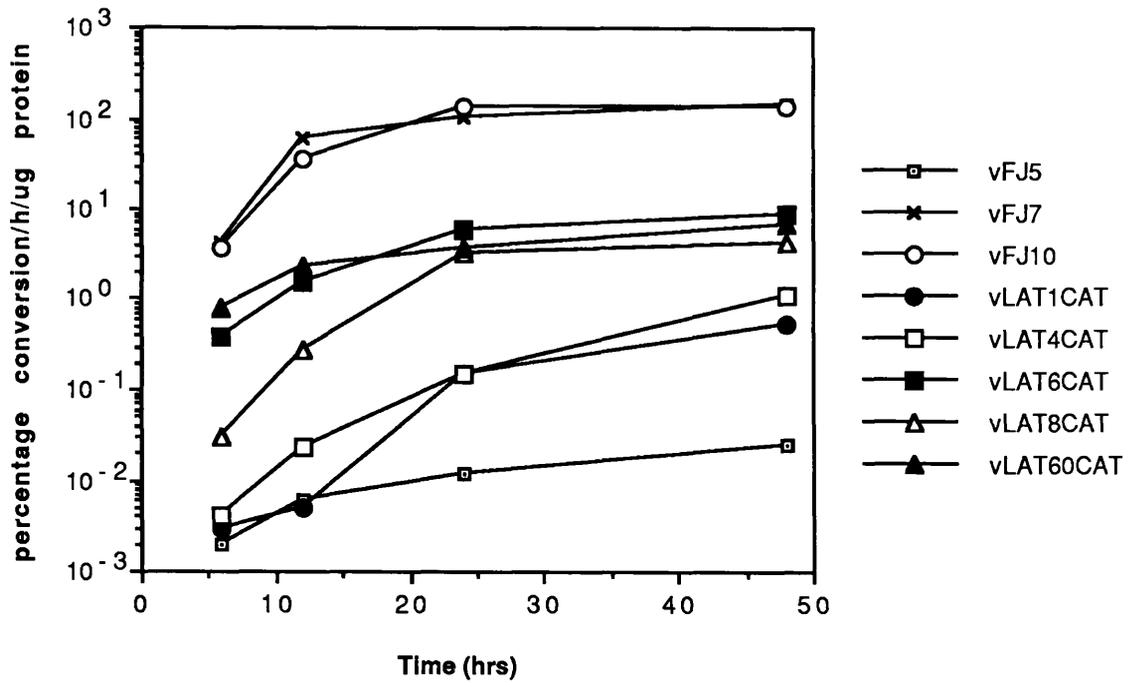
3.2. C1300 Cells. Since the LAT gene is the only HSV-1 gene active during latency in neuronal cells, it was considered interesting to determine the behaviour of the LATCAT viruses in neuronally-derived cell lines. Short term expression assays in neuronal cells have proven difficult in the past due to their low susceptibility to standard transfection procedures. In this respect, the 1802/1804 vectors provide ideal vehicles for introducing promoter/reporter gene fusions into neuronal cells for such assays. Unfortunately, the amount of material available from primary cultures of sensory neurons were insufficient for such experiments. In this study, the C1300 mouse neuroblastoma cell line (August-Tocco & Sato, 1969) has therefore been employed. This cell line has been reported to be non-permissive for lytic infection with HSV (Vahlne & Lycke, 1977, 1978), an effect mediated by failure at the level of viral immediate-early gene transcription (Kemp & Latchman, 1989; Kemp *et al.*, 1990).

As in the BHK cells above, the levels of CAT activity produced by the LATCAT viruses together with vFJ5, vFJ7 and vFJ10 were determined at 6, 12, 24 and 48 hours post infection with a moi of 5 at 37°C. A typical set of data is shown on Figure 32, which again, for sake of clarity, is presented on both logarithmic and linear plots. Figure 32(a) shows that the HSV lytic cycle promoters maintain their 1-3 log superiority over the LATCAT constructs, and that this activity is similar to that exhibited in BHK cells (although direct comparison of promoter activity in different cell lines is difficult). This activity displayed by the lytic cycle promoters, and the IE-4/5 promoter in particular, is intriguing since at up to 6 hours pi, Kemp & Latchman, (1989) could detect virtually no IE mRNA in HSV-1 infected C1300 cells. The IE-

Figure 31. CAT activities produced by recombinant 1804-derived LAT promoter/CAT fusion viruses at various times after infection of BHK cells. a) CAT activities generated by vFJ5, vFJ7, vFJ10, vLAT1CAT, vLAT4CAT, vLAT6CAT, vLAT8CAT and vLAT60CAT shown on a logarithmic scale. b) CAT activities generated by the LAT promoter viruses alone, shown on a linear scale. CAT activities were determined at 6, 12, 24 and 48 h post infection and expressed as the percentage [¹⁴C]chloramphenicol converted per h per μ g protein. The symbols representing each virus are indicated in the legends beside each graph.

a)

BHK Cells



b)

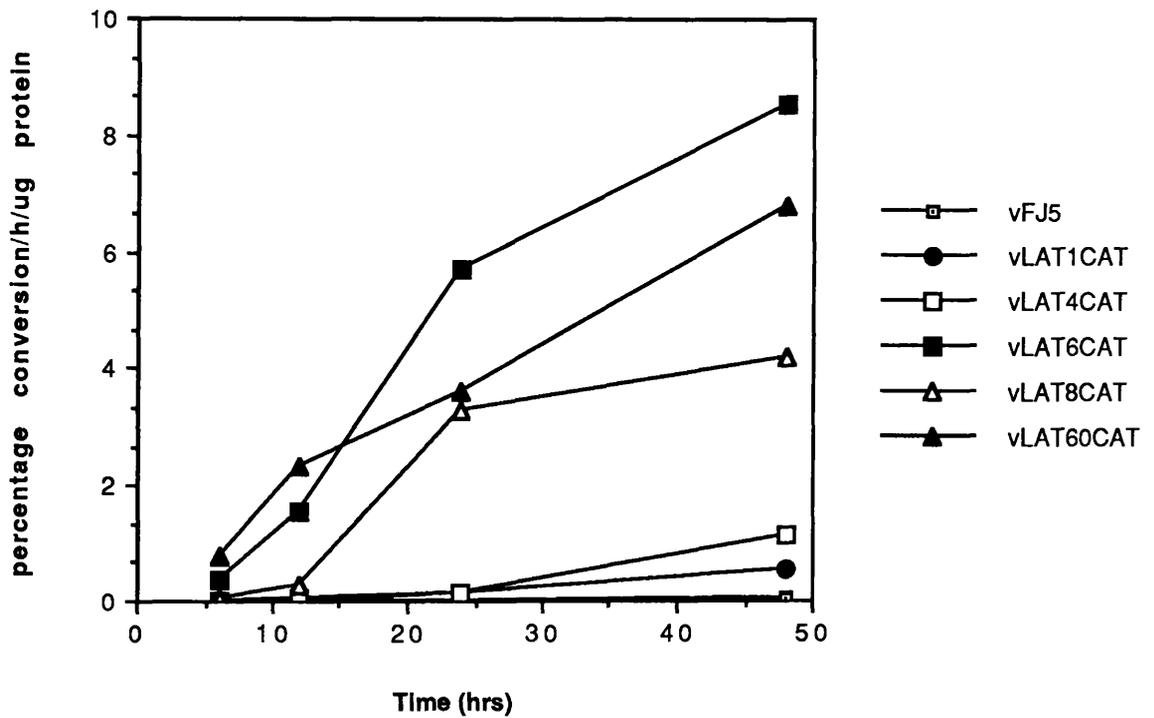
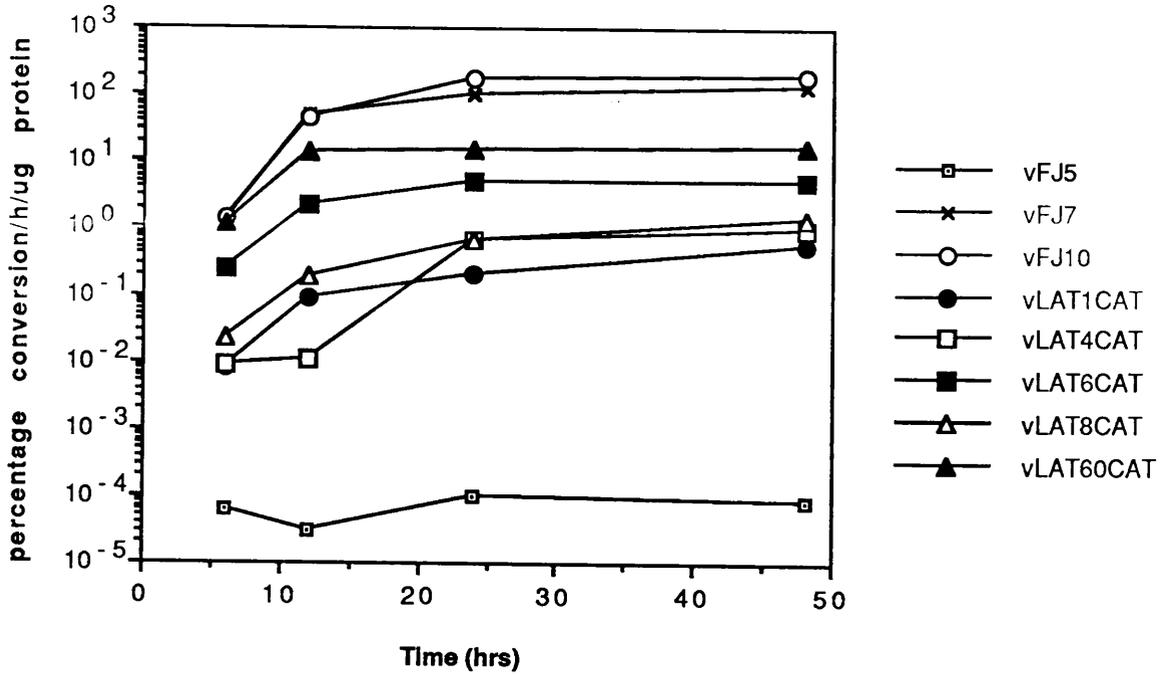


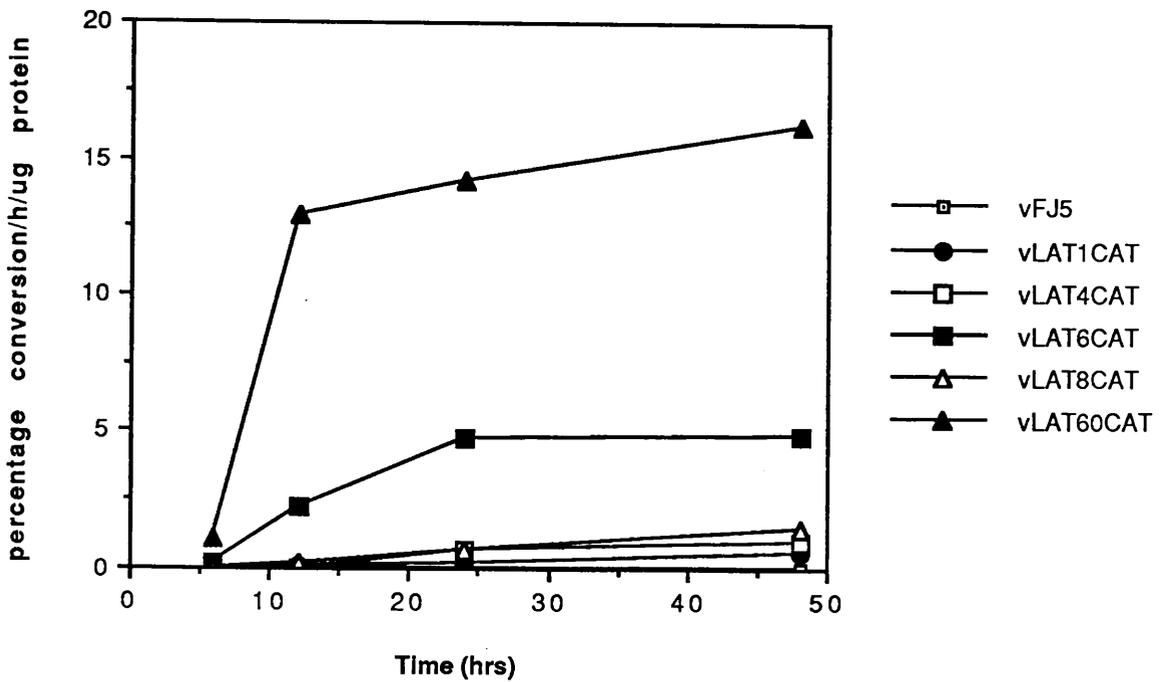
Figure 32. CAT activities produced by recombinant 1804-derived LAT promoter viruses at various times after infection of neuroblastoma C1300 cells. a) CAT activities generated by vFJ5, vFJ7, vFJ10, vLAT1CAT, vLAT4CAT, vLAT6CAT, vLAT8CAT and vLAT60CAT shown on a logarithmic scale. b) CAT activities generated by the LAT promoter viruses alone, shown on a linear scale. CAT activities were determined at 6, 12, 24 and 48 h post infection and expressed as the percentage [¹⁴C]chloramphenicol converted per h per µg protein. The symbols representing each virus are indicate in the legends beside each graph.

a)

C1300 Cells



b)



4/5 promoter activity observed here could be explained as a result of a higher moi overcoming the non-permissivity of C1300 cells. Indeed, Kemp & Latchman (1989) demonstrated that increasing the amounts of virus and the incubation time, led to a majority of cells staining with anti-Vmw175 antibody by 48h pi.

The pattern of CAT activity exhibited by the LATCAT viruses (Figure 32(b)), shows a strikingly different pattern to that observed in BHK cells. While the relative activities displayed by vLAT1CAT, vLAT4CAT, vLAT8CAT and vLAT6CAT are essentially similar to those in BHK cells, the activity exhibited by vLAT60CAT is at least 3 times greater than vLAT6CAT. This contrasts with the results from BHK cells, and demonstrates that sequences upstream of the LAT6 clone (perhaps those in conserved loci 1 and 2), while not contributing to LAT promoter activity in BHK cells, enhance LAT promoter activity in neuroblastoma cells, and may therefore contain elements which contribute to neuronal specific expression of the LAT gene.

3G. DISCUSSION

3G.1. Identification of the LAT Promoter

A prerequisite to understanding the seemingly complex nature of LAT gene expression is the mapping of the basic promoter elements necessary for LAT promoter function. To this end, this study has employed a combination of *in vitro* and *in vivo* expression assays based on sequence analysis to define upstream regions of the LAT gene capable of functioning as a promoter when fused to the CAT reporter gene.

Sequence analysis has demonstrated that, in agreement with other investigations, (Wechsler *et al.*, 1988, 1989) the first identifiable RNA polymerase II promoter elements comprising TATA and CAAT box homologies

and two potential Sp1 binding sites occur approximately 700bp upstream of the mapped 5' end of LAT. These putative promoter elements occur in regions of HSV-1/HSV-2 homology, while the region immediately upstream, and indeed, downstream of the 5' end of LAT shows no appreciable homology whatsoever (Figures 6 and 7). The HSV-1/HSV-2 homologies comprise 9 regions of conserved loci, mostly centred around and upstream of the putative promoter elements, while only 3 loci are located downstream of the TATA box. Of the known promoter elements themselves, only the TATA box is perfectly conserved in the HSV-2 sequence (Figure 7).

Results from the short term transfection assays (section 3C) concur with an accumulating body of evidence (Javier *et al.*, 1988; Dobson *et al.*, 1989; Steiner *et al.*, 1989; Batchelor & O'Hare, 1990; Zwaagstra *et al.*, 1990, 1991; Devi-Rao *et al.*, 1991) that the determinants of LAT gene expression are indeed located around the putative RNA pol II promoter region. Data from the transfection assays (Tables 3 to 6) demonstrate that the main determinants of *in vitro* promoter activity map to a 279 bp region between -940 and -661 (the LAT6 clone) containing all of the putative promoter elements, thereby agreeing with Zwaagstra *et al.* (1989) who independently used the same construct in Vero cells. The transfection assays with pLAT1CAT, pLAT2CAT and pLAT3CAT (Table 3) demonstrate that deletion from the *HinfI* site of the LAT1 clone (-940 to +151) to the *PstI* site at -798 (LAT2), thereby removing the CAAT and Sp1 homologies, reduces promoter activity 7-fold. Promoter activity is virtually abolished on further deletion to -595 (LAT3) which removes the TATA box. This, together with the LAT6 (-940 to -661; Table 4) result which maps the right hand site of the LAT promoter demonstrates that the determinants of core promoter activity map to the 137bp region flanked between the *PstI* site at -798 and *PvuI* site at -661.

In their study, Batchelor & O'Hare, (1990), using a similar strategy in HeLa cells, mapped the main determinants of LAT promoter activity to the 140bp region between the *Pst*I site at -798 and *Pvu*I site at -659, (their clone being constructed differently to LAT6), and showed that this activity increased only marginally (2-fold) on addition of sequences upstream to a *Nae*I site at -980. They also observed that the *Nae*I fragment spanning -251 to +201, similar to the LAT5 clone, and in agreement with the results in Table 3, exhibited negligible constitutive promoter activity. Devi-Rao *et al.* (1991), also employing a similar strategy, but in rabbit skin cells, noted that a 352bp *Eag*I fragment (-1022 to -670; roughly equivalent to LAT6) exhibited a 5-fold higher activity than a 1063bp *Eag*I/*Pss*I fragment (-1022 to +41; roughly equivalent to LAT1). Similarly, pLAT6CAT exhibited a 9 to 11-fold higher activity than pLAT1CAT in BHK cells (Tables 3 and 6). They also demonstrated that a 636bp *Pst*I/*Pss*I fragment (-595 to +41; similar to LAT3) exhibited negligible activity in the neuronal derived cell line Neuro2A.

While the results of Table 6 show that an additional 519bp upstream from the *Hin*fI site at -940 (LAT60; -1459 to -661) marginally increases LAT promoter activity (by 50%), Zwaagstra *et al.*, 1990) showed that clones with an additional 331bp (-1271 to -662) and 1652bp (-2592) fused to their version of LAT6 reduced promoter activity by 3-fold in BHK cells and mouse L-cells and 6-12 fold in Vero and CV-1 cells. Batchelor & O'Hare, (1990) however, found that upstream sequences extending back to -1267 from the *Nae*I site at -979, effected a minor decrease in activity. These results, as a whole, indicate that sequences upstream from the *Hin*fI site at -940 can exert only a minor influence (depending on laboratory and cell line), upon LAT promoter activity in non-neuronal derived cell lines.

In terms of promoter strength, the constitutive

activity displayed by the LAT6 promoter was approximately 2.3 times that of the HSV-1 gD promoter, though 5 times weaker than the HSV-2 IE-4/5 promoter. Batchelor & O'Hare, (1990), showed that their PvuI (-1267 to -650) fragment promoter was at least as strong as the SV40 enhancer/promoter and HSV-1 IE-1 and IE-3 promoters, while Devi-Rao *et al.* (1991) showed that their equivalent of LAT6 (the *EagI* -1022 to -671 fragment) was 10-fold stronger than the HSV-1 dUTPase (U_L50) promoter.

While the evidence described above demonstrates that the putative promoter region is functional during *in vitro* expression assays, the most compelling evidence that this region comprises the *bona fide* LAT promoter comes from *in vivo* studies with recombinant viruses. Dobson *et al.* (1989) showed that a β -globin gene, when inserted 19bp downstream of the TATA homology at position -670, selectively expressed β -globin mRNA in latently infected sensory neurons. They also showed that a second recombinant virus deleted for the 203bp *PstI* region (and therefore the TATA box), abolished any apparent LAT gene expression. Steiner *et al.* (1989) showed that HSV-1 variant 1704 which is deleted for one complete copy of the LAT gene promoter and 5' portion of the other copy (-2559 to +564 and -1235 to -293 respectively) also failed to express detectable levels of LAT during latency in the mouse. Similarly, Javier *et al.* (1988) with HSV-1 variant X10-13 which harbours a deletion of the LAT promoter (3' terminus between -263 and -225) is also unable to express the LAT gene. All current evidence therefore demonstrates that the *bona fide* LAT promoter is located in this upstream location rather than adjacent to the mapped 5' end of LAT.

The finding that the LAT promoter was located in this upstream location relative to the mapped 5' end of the 2kb LAT suggested that the LAT transcription pattern was more extensive than previously thought. While earlier mapping studies of the LAT transcript had scored the region

between the TATA box and 5' end of LAT as negative, it became increasingly apparent that the presence of very faint *in situ* signals (Wechsler *et al.*, 1988b) were indicative of an unstable or low abundance transcript in this region. Subsequent work (Dobson *et al.*, 1989; Mitchell *et al.*, 1990a; Zwaagstra *et al.*, 1990), demonstrated that these additional weak hybridisation signals corresponded to a poly(A)⁺ 8.3kb transcript (m-LAT) extending from the vicinity of the TATA box to a polyadenylation site immediately downstream from the 3' end of the IE-3 gene. The 5' end of m-LAT was mapped by primer extension (Zwaagstra *et al.*, 1990) to approximately 28bp downstream of the first T of the TATA box at position -659 (...CGATCGCG...). Devi-Rao *et al.* (1991) when performing RNase protection analysis of poly(A)⁺ RNA from HSV-1 infected cells mapped the 5' end of m-LAT to approximately position -657 (...CGATCGCG...). They also showed that the 2 kb LAT is uncapped and maps between 5' splice donor and 3' splice acceptor 1950 bp apart, and this, together with the results of Farrell *et al.* (1991) who showed that the 2 kb LAT can be spliced out of a β -gal/LAT fusion, confirmed earlier suspicions that the poly(A)⁻ LAT is a stable intron derived from the splicing of a large primary transcript. The presence of a spliced polyadenylated product of this splicing reaction, ie 6.3kb, has however, yet to be demonstrated.

Comparison of the HSV-1 and HSV-2 LAT sequence (Figures 6 and 7), fails to demonstrate any homology indicative of the presence of conserved reading frames. This view is engendered by the lack of any ATG codons in the HSV-2 m-LAT sequence between the TATA box and corresponding 5' end of the 2 kb LAT (the HSV-1 sequence has 3 ATG's). While it seems that the LATs, as a whole, do not code for any proteins, it is almost certain that they must possess some subtle, although perhaps critical role in the pathogenesis of natural latent infections. While the

characterisation of any processed forms of the primary poly(A)⁺ LAT (possibly by the cloning of associated cDNAs) will be of interest *per se*, it seems that any search for an associated LAT polypeptide will not prove fruitful.

The observation in section 3C.4 that deletion of sequences downstream of the primary LAT RNA cap site can affect CAT activity was particularly intriguing. While the 90 bp deletion (-341 to -251) in plasmid p Δ 1CAT (Figures 9 and 17) which removes conserved locus 8 had no apparent effect on CAT activity, the activity exhibited by plasmids p Δ 2CAT, p Δ 3CAT and pLAT4/5CAT was significantly greater than that exhibited by pLAT1CAT. Since the effect generated by these deletions was generally similar, it was interesting to note that the only similarity in the respective deletions is a 54bp sequence between the *Sma*I site at -395 and *Nae*I site at -341, a region encompassing conserved locus 7. If conserved locus 7, and indeed, 8 and 9 are involved in LAT gene regulation, their location approximately 300 bp downstream of the m-LAT cap site makes it difficult to envisage how they could directly influence events occurring at the LAT promoter. It is possible that their influence on CAT activity is exerted at the translational level or in mRNA stability or processing, however, it is difficult to envisage how this relates to *in vivo* LAT expression since it appears that LATs may not code for any polypeptides. Indeed, the observation by Devi-Rao *et al.* (1991), that a virus (8117) with an 897 bp deletion 360 bp downstream from the primary transcript cap site which does not express the 2 kb LAT, but still expresses mLATs, indicates that sequences downstream from the cap site have no critical role to play in LAT gene expression. The level at which these deletions exert their influence could be differentiated by performing primer extension, Northern or S1 assays to measure the *in vitro* level of transcription and comparing them with CAT activity. Another possibility is, of course, that the apparent increase in

CAT activity is generated by artefactual means. Inspection of the LAT1 sequence reveals that it contains 3 ATG codons (at -442, -279 and -173; one in each frame), and that the reading frames specified by each are terminated by stop codons within the LAT1 sequence (at -276, +69 and +7 respectively). It is a distinct possibility that these reading frames interfere with translation of the CAT reading frame, and that the removal of ATGs in plasmids p Δ 2CAT, p Δ 3CAT and pLAT4/5CAT could relieve this interference, thereby allowing increased translation of the CAT reading frame and therefore giving rise to increased levels of CAT activity. This possibility could be examined by removal of the ATG's from the LAT1 sequence by site directed mutagenesis, or alternatively, by generating similar constructs from the corresponding HSV-2 sequence which lacks ATG codons.

The existence of these conserved loci in the primary LAT transcript which is otherwise devoid of HSV-1/HSV-2 homology, seems indicative that, like those upstream around the LAT promoter, they are of functional significance. It will be interesting to see if future studies prove this to be the case.

3G.2. Regulation of the LAT Promoter

To assess LAT promoter response to HSV-1 regulatory proteins, BHK cells transfected with the LATCAT plasmids were infected with virus. The gD and IE-4/5 promoters exhibited a characteristic induction (160-fold and 10-fold respectively) expected as their response to regulation *in trans* by virus encoded factors. The LATCAT plasmids exhibited a generally low, but positive response to virus infection. The infected cell CAT activities exhibited by pLAT1CAT, pLAT2CAT, and pLAT3CAT (Table 3), confirm that the reporter elements responsible for promoter activity in the LAT1 clone are upstream of the *Pst*I site at -595; while the level of induction of pLAT2CAT and pLAT3CAT were

relatively high (11 and 16-fold respectively), the levels of CAT activity attained by these plasmids were still very low (approximately 2.5 and 10-fold less than that exhibited by pLAT1CAT respectively). Of the clones centred around the LAT promoter region itself, (pLAT4CAT, pLAT6CAT, and pLAT8CAT), pLAT6CAT exhibited the greatest response (9.2 to 11.8-fold; Tables 4 and 6), compared with 3.14-fold for pLAT4CAT and 4.7-fold for pLAT8CAT. The increase in response between the LAT4, the LAT8 and the LAT6 clones could be the result of a distance effect such that as the distance between the reporter gene and the promoter elements decreases, the level of response to infection increases. Also, since the level of LAT6 induction is twice that of LAT8 induction, it is possible that the difference in response to HSV-1 infection between these clones is due to the presence of a negative regulatory element, the putative Vmw175 binding site perhaps, (section 3C.6; see below) which confers a negative regulatory effect on LAT promoter activity during the HSV-1 lytic cycle. The observation that the addition of upstream sequences to the LAT6 clone (in LAT60) does not lead to any significant change in response to HSV-1 infection confirms that the sequences elements responsible for LAT promoter induction are confined to the LAT6 clone.

The most intriguing results of the infected transfection assays are those obtained with clones (pLAT5CAT, pLAT7CAT, and pLAT4/5CAT) containing sequences between +151 and +201 of the LAT sequence. The LAT5 clone (-251 to -201) which exhibited negligible constitutive CAT activity, displayed a relatively high response to viral infection (112.5-fold; Table 3). A similar kind of response, though not quite as high, was observed with the LAT7 and LAT4/5 clones (Tables 4 and 5 respectively). Indeed, the superinfected CAT activity exhibited by pLAT4/5CAT was the highest observed for any of the LATCAT plasmids. These observations suggest that the 150bp region

between +151 and +201 of the LAT sequence harbours an regulatory that exerts an influence on LAT promoter activity during the HSV-1 lytic cycle. Examination of this region of the LAT sequence, however, fails to reveal any obvious relationship with known sequence motifs, and indeed, this particular region is not highly conserved between the HSV-1 and HSV-2 sequences.

While Devi-Rao *et al.* (1991), found that their LAT promoter clones behave in a similar fashion to those described here, with the LAT6 equivalent (the -1022 to -671 *EagI* fragment), being stimulated 3.5-fold, Batchelor & O'Hare (1990) showed that promoter activity exhibited by their LAT clones (the -1267 to -658 *PvuI* fragment and -797 to -595 *PstI* fragment) after infection was 4-5 fold lower than observed in mock-infected cells. These conflicting results are perhaps a consequence of cell-specific effects; Batchelor & O'Hare (1990), used Hela cells for their assays, while Devi-Rao *et al.* (1991) used rabbit skin cells.

To examine regulation of the LAT promoter by individual HSV-1 regulatory proteins, plasmids pLAT6CAT, pLAT8CAT and pgDCAT were co-transfected with plasmids p111 and p175, specifying Vmw110 and Vmw175 respectively, both alone and in combination. While the control pgDCAT plasmid exhibited a characteristically positive response when co-transfected with either p111 or p175 and synergistic response when co-transfected with both plasmids, pLAT6CAT, by contrast, exhibited a weak response. PLAT6CAT was stimulated 1.7-fold with p111 and 2.2-fold with p175, in agreement with Zwaagstra *et al.* (1989), who performed similar experiments on an identical clone in Vero cells. In addition, both Zwaagstra *et al.* (1989) and Batchelor & O'Hare (1991) showed that despite the presence of a sequence bearing similarity to the Vmw65-response TAATGARAT consensus element, their LAT promoter constructs exhibited no significant increase in activity when co-transfected

with a Vmw65-expressing plasmid.

Most significantly, however, was the demonstration that the activity exhibited by the LAT8 clone was repressed by Vmw175 (Figure 19). A dose response curve which titrated pLAT6CAT, pLAT8CAT and pIE-3CAT activity against increasing amounts of p175 demonstrated that this repression is as efficient as Vmw175-mediated autoregulation of the IE-3 promoter, and that this effect must be mediated by sequences between the 3' ends of the LAT6 and LAT8 clones. This finding is in complete agreement with Batchelor & O'Hare (1990) who showed that the level of LAT promoter repression was even greater than that observed due to the autoregulatory effect of Vmw175 on its own expression. LAT repression by Vmw175 is most likely mediated through a possible Vmw175 binding homology located around the PvuI site at -661. The LAT6 clone is truncated for this homology, with its 3' end immediately adjacent to this motif, thus explaining why pLAT6CAT, like the identical clone of Zwaagstra *et al.* (1989) is actually induced, albeit weakly, by Vmw175. Batchelor & O'Hare (1990) found that their PvuI clone (-1267 to -658) was repressed by Vmw175, however, the central AT of the PvuI site in their clone was retained, and when fused to their vector sequences, reformed a Vmw175 binding homology. The final proof that Vmw175 does interact with this ATCGTC homology in the LAT sequence will come from gel retardation experiments, however, it is interesting to note that, like the IE-gene Vmw175 binding sites, the putative binding site in the LAT gene is located at the mapped cap site of the primary LAT transcript (Zwaagstra *et al.*, 1990; Devi-Rao *et al.*, 1991).

Data from the transfection assays demonstrated that the LAT8 promoter clone is induced 4.7-fold following infection with virus (Table 4). In addition, when introduced into the virus vector genome, CAT activity exhibited by vLAT8CAT steadily rose during infection of

both BHK and C1300 neuroblastoma cells. While it has been shown that Vmw175 can repress the IE-1, 3 and 4/5 promoters (Gelman & Silverstein, 1987a, b; Paterson & Everett, 1988a, b; Resnick *et al.*, 1989), and now the LAT promoter (Batchelor & O'Hare, 1990; this study), during transfection assays, it has only ever been convincingly shown for the IE-3 promoter during virus infection (DeLuca & Schaffer, 1988). Indeed, Everett & Orr, (1991), demonstrated that a mutated Vmw175 binding site in the IE-1 promoter which destroyed the ability of Vmw175 to bind the site, and greatly reduced the ability of Vmw175 to repress the IE-1 promoter in transfection assays, had no effect on the level of Vmw110 expression during normal HSV-1 infection. It seems that the direct repression of IE and LAT promoters by Vmw175 is not a major feature of viral growth in tissue culture, though it may have an important role in some other aspect of the virus life cycle. Batchelor & O'Hare (1990), proposed that the Vmw175-mediated repression of the LAT gene may be responsible for the apparent decrease in LAT expression observed during virus reactivation in explantation and cocultivation experiments (Spivack & Fraser, 1988b).

3G.3. Virus Vector Experiments

While transfection assays are clearly useful for mapping promoters, and providing an insight as to how they are regulated, a proper understanding of the LAT promoter can only come from its characterisation in the context of the HSV-1 genome during lytic infection of tissue culture cells, and in animal latency systems. To this end, LAT promoter/reporter gene constructs were introduced into the 1802 and 1804 HSV-1 vectors. The inherent advantage of this system over other virus-based studies (Javier *et al.*, 1988; Dobson *et al.*, 1989; Steiner *et al.*, 1989) is that it allows the definitive characterisation of promoters by defining both the 5' and 3' ends of promoter fragments, and

thereby the minimal promoter sequences capable of LAT promoter function.

The results of sections 3D.4 and 3F.3 demonstrated that the LAT promoter clones behaved in the same manner relative to each other in the context of the virus genome during infection of tissue culture cells as they did during the infected transfection assays. This is an important validation of the findings from the transfection experiments which are prone to anomalous results due to the inconsistent nature of the short term transfection assay.

Perhaps the most significant result to come from the vector experiments was the differential activities of the LAT6 and LAT60 clones observed during lytic infection of BHK and C1300 neuroblastoma cells. While infection of BHK cells showed that, as observed from the infected transfection assays, CAT activity exhibited by vLAT60CAT is marginally less than that exhibited by vLAT6CAT (Figures 31 and 32), vLAT60CAT activity in C1300 neuroblastoma cells is more than three times that exhibited by vLAT6CAT, thereby suggesting that sequences upstream of the *Hinf*I site at -940 may confer neuronal specificity to the LAT promoter. Batchelor & O'Hare (1990) showed that in transfection assays, their *Pvu*I clone (-1267 to -658) was eight times stronger than a *Pst*I/*Pvu*I clone (-797 to -658) in IMR-32 neuroblastoma cells while being only twice as strong in HeLa cells, thus concluding that the upstream -1267 to -797 region is selective for promoter activity in neuroblastoma cells. Similarly, Zwaagstra *et al.* (1991), showed that sequences upstream of the *Hinf*I site could increase activity up to 3-fold in immortalised neurons and neuroblastoma cells. From examination of the LAT sequence, it would seem that the most likely location of the motifs responsible for selective expression in neuronal-derived cell lines is in conserved loci 1 or 2, however, this can only be verified by more precise studies.

Now that the LAT promoter has been clearly mapped,

further characterisation of it is required to identify specific motifs and associated binding factors which, particularly in neuronal cells, could contribute to selective expression of the LAT gene during latency. A step in this direction has been taken by Zwaagstra *et al.* (1991), who, using DNA footprinting and gel retardation analysis to identify regulatory sequences in the -940 to -659 *HinfI/PvuI* region, identified a LAT promoter binding factor (LPBF) which bound a region that includes the sequence CCACGTGG located at -731 to -724. Deletion of this sequence causes a significant reduction in promoter activity in all neuronal and non-neuronal cell lines, and it is therefore probably required for maximum constitutive activity of the LAT promoter. Interestingly, this LPBF sequence is one of those identified in section 3B.4 as having significant homology to a CCAGG motif found in a number of neuronal specific genes. The LPBF sequence, though is not well conserved in the HSV-2 ; (..CC-CGTG-..)(see Figures 7 and 8). Gel shift assays with a probe containing the upstream homology located at -915 to -911 did not identify any prominent bands, though some subtle differences were seen when comparing HeLa and C1300 extracts (Zwaagstra *et al.*, 1991). Further analysis by Leib *et al.*, (1991) has demonstrated the presence of a functional cAMP-response element at position -704 to -698 (..TGCGTCA..), the mutation of which results in a complete loss of cAMP-mediated inducible stimulation, and a 4-fold reduction in basal activity.

The next stage in characterisation of the LAT promoter is to apply the 1804 vector system to animal latency systems. An ideal strategy for this is to replace the CAT reporter gene with the the *lacZ* gene, thus allowing the use of histochemical analysis to allow detection of promoter activity in individual cells in latently infected tissues. Due to lack of time, however, this aspect of the study of the LAT promoter was unable to be carried out,

though this should ultimately allow a definitive mapping of the minimal 5' and 3' ends of the LAT promoter necessary for LAT gene expression in neuronal cells during latency.

CHAPTER 4

CHARACTERISATION OF THE HUMAN NEUROFILAMENT GENE (HNF-L) PROMOTER

4A. Introduction

An intriguing question regarding HSV latency in the neuron is whether or not the promoter of an endogenous neuronal gene will function correctly in the context of the latent virus genome. To date, only the LAT and MMTV LTR (Dobson *et al.*, 1990) promoters have been shown to be active in the HSV-1 genome during latency. To this end, a clone comprising the coding and 5' flanking sequences of the human neurofilament-light chain (HNF-L) gene was acquired (kindly supplied by K. Yazdanbakhsh).

Neurofilaments (NF's) are major cytoskeletal elements of nerve cells and are believed to play an important role in the control of axon calibre and maintenance of cell shape (Morris & Lasek, 1982; Hoffman *et al.*, 1984; reviewed by Julien & Grosveld, 1991). NF proteins have also been widely used as markers of nerve cells, with their neuronal specificity being confirmed by the use of cloned DNA probes in *in situ* analysis (Leisi *et al.*, 1986; Zopf *et al.*, 1987). NF's belong to the multigene family of intermediate filament (IF) proteins which consists of a number of ubiquitous and cell specific proteins including vimentin in cells of mesenchymal origin, desmin in muscle cells, glial fibrillary acid protein (GFAP) in glial cells and keratins in epithelial cells (reviewed by Lazarides *et al.*, 1982; Osborn & Weber, 1982). NF's are made by copolymerisation of three proteins with apparent molecular weights on SDS gels of 68,000 (NF-L), 150,000 (NF-M) and 200,000 (NF-H) (Julien & Grosveld, 1991).

The 5' end of the HNF-L gene, like those of the other NF genes, contains a GC rich methylation free region

suggesting that the promoter region may be accessible to transcription in all tissues, and that nerve cell specific expression of NF genes may be achieved, in part, by suppression in non-nerve cells (cited in Julien & Grosveld, 1991). All three NF genes have a proximal TATA box resulting in a single transcription initiation site approximately 30 bp downstream (Julien *et al.*, 1987a). Other upstream elements which were identified by sequence analysis included a complementary CAAT box homology ("GATCGATC") located 35 bp upstream of the TATA box, and a complementary Sp1 binding site consensus ("ACCCGCCTT") located 110bp upstream from the TATA box (Julien *et al.*, 1987b). The sequence of the HNF-L gene, published in Julien *et al.*, (1987b), extends upstream to only -160bp from the HNF-L gene cap site.

4B. Strategy

To characterise the promoter of the HNF-L gene, two 5' flanking sequences extending from an *Xma*III site at +84 to *Bam*HI sites at -296 and -896 were purified and fused to the CAT gene in pFJ5. The *Xba*I fragments from these plasmids were isolated and purified, and introduced into the genome of the virus vector. The plasmids and viruses were then used for characterisation of the HNF-L promoter in transfection assays, and during lytic infection of tissue culture cells respectively. A diagrammatic representation of the fragments used for characterisation of the HNF-L promoter can be seen on Figure 33.

4C. CHARACTERISATION OF THE HNF-L PROMOTER IN TRANSFECTION ASSAYS

4C.1. Construction of HNF-L Promoter/CAT Plasmids

pNF1CAT Plasmid pNF1CAT contains a 380 bp fragment of HNF-L sequence extending from a *Xma*III site at +84 to a *Bam*HI site at -296. The *Xma*III site in pHNF-L (see Figure 33) was

converted to a *Bgl*III site by *Xma*III digestion, blunt-ending with T4 DNA polymerase and ligation of the *Bgl*III linker 5'-CAGATCTG-3', thereby generating pHNF-L-*Xma*III+*Bgl*III. The *Bam*HI/*Bgl*III fragment from pHNF-L-*Xma*III+*Bgl*III was isolated and purified, then ligated into *Bgl*III digested pFJ5 DNA. Clones with the appropriately orientated fragment were selected for by restriction enzyme digestion and designated pNF1CAT. Diagnostic restriction profiles of these plasmids are shown on Figure 34.

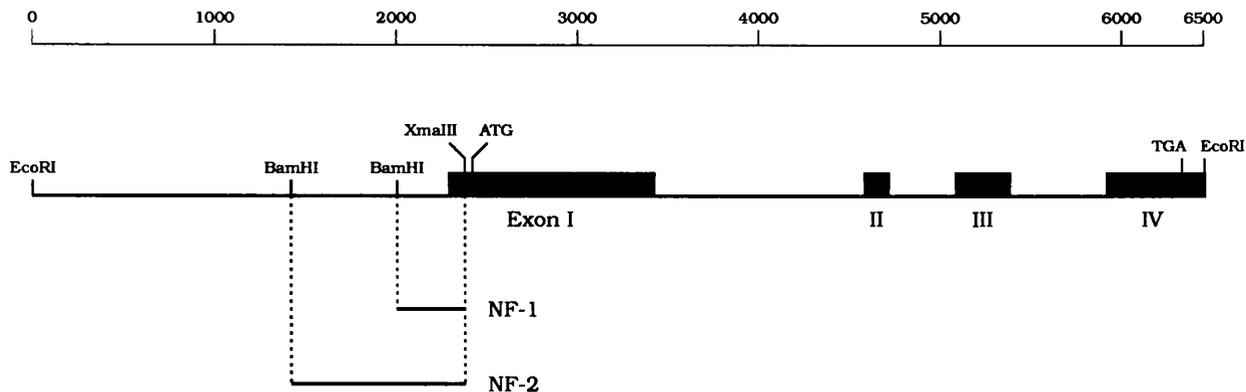
pNF2CAT Plasmid pNF2CAT contains a 980bp fragment of HNF-L sequence extending from the *Xma*III site at +84 to the *Bam*HI site at -896. Plasmid pHNF-L-*Xma*III+*Bgl*III was partially digested with *Bam*HI. The linearised plasmid was further digested with *Bgl*III and the appropriate *Bam*HI/*Bgl*III fragment isolated and purified, then ligated into *Bgl*III digested pFJ5. Clones with the appropriately orientated fragment were selected for by restriction enzyme digestion and designated pNF2CAT. Figure 34 indicates the presence of an *Xba*I site approximately 150 bp downstream from the 5' end of the NF2 sequence. This sequence from the 5' end of the NF2 clone will therefore be missing when the *Xba*I fragment is purified from pNF2CAT for ligation into the virus vector (section 4D.1).

4C.2. Transfection Assays

The promoter activity of the pNFCAT constructs were determined by quantitation of CAT activity in transfection assays. 3µg of each plasmid was transfected into BHK monolayers, and after 24h, extracts were prepared and assayed for CAT activity. The results shown in columns 2 and 3 of Table 8 compare the activity of the pNFCAT constructs to that of the HSV lytic promoters in pFJ10 and pgDCAT. As in section 3C, the data has been normalised with respect to the CAT activity exhibited by pLAT1CAT. The data for pLAT1CAT is not shown, but the transfection assays for

Figure 33. Schematic representation of NF promoter fragments. The top line gives the scale of the Figure in bp. The second line shows the main features of the pHNF-L clone which was obtained as an *EcoRI* fragment cloned into the *EcoRI* site of pUC18. The exons of the NF-L gene are shown as dark boxes, with the positions of the ATG and TGA initiation and termination codons indicated. The two fragments used for characterisation of the NF promoter are shown below. The NF1 fragment extends from -296 to +84 and the NF2 fragment from -896 to +84 relative to the cap site of the NF-L gene.

Table 8. Relative CAT activities exhibited by NF promoter plasmids. The CAT activities exhibited by the NF promoter plasmids together with plasmids pgDCAT and pFJ10 were determined and calculated relative to the CAT activity exhibited by pLAT1CAT which was given the nominal value of 1 (data not shown). The mean of at least four independent determinations is given (column 2) with the standard error of the mean (SEM) (column 3). Column 4 records the relative CAT activity exhibited upon infection of transfected cells with 5pfu/cell HSV-1 17+ for 18h, with the SEM given in column 5. The level of induction of CAT activity upon infection, relative to uninfected cell CAT activity is given in column 6.



| Plasmid | Relative CAT Activity | | | | Induction |
|---------|-----------------------|------------------|----------|------------------|-----------|
| | Uninfected | Std. Error (+/-) | Infected | Std. Error (+/-) | |
| pFJ10 | 55.91 | 10.9 | 582.37 | 89.59 | 10.4 |
| pgDCAT | 3.4 | 0.59 | 555.91 | 90.26 | 163.5 |
| pNF1CAT | 3.6 | 0.14 | 484.9 | 43.2 | 132.8 |
| pNF2CAT | 6.8 | 0.85 | 649.6 | 48.5 | 95.5 |

Figure 34. Restriction digest demonstrating integrity of the NF promoter plasmids. Plasmids pNF1CAT (lane 1) and pNF2CAT (lane 2) were digested with *Hind*III and *Xba*I and run on a 1% agarose gel along with 123 bp size markers (lane 3). The top two fragments of pNF1CAT and pNF2CAT represent vector sequences. The third and fourth fragments co-migrating in lanes 1 and 2 respectively are the 797bp *Hind*III fragment containing the CAT gene. The *Hind*III/*Xba*I NF1 promoter fragment migrates the correct distance consistent with its expected size. The NF2 promoter fragment, however, runs slightly smaller than expected. The presence of an additional band marked (X) in lane 2 indicates the presence of an *Xba*I site approximately 150bp downstream from the 5' end of the NF2 clone.

the NF/CAT and CAT promoter/CAT fusion plasmids described in section 3.1 will be carried out at the same time.

The results demonstrate that both pNF2CAT clones perform well as promoters in this assay, exhibiting at least as much activity as pCAT. The activity exhibited by pNF2CAT is in fact that in NIH 3T3 cells is 20% of the activity.

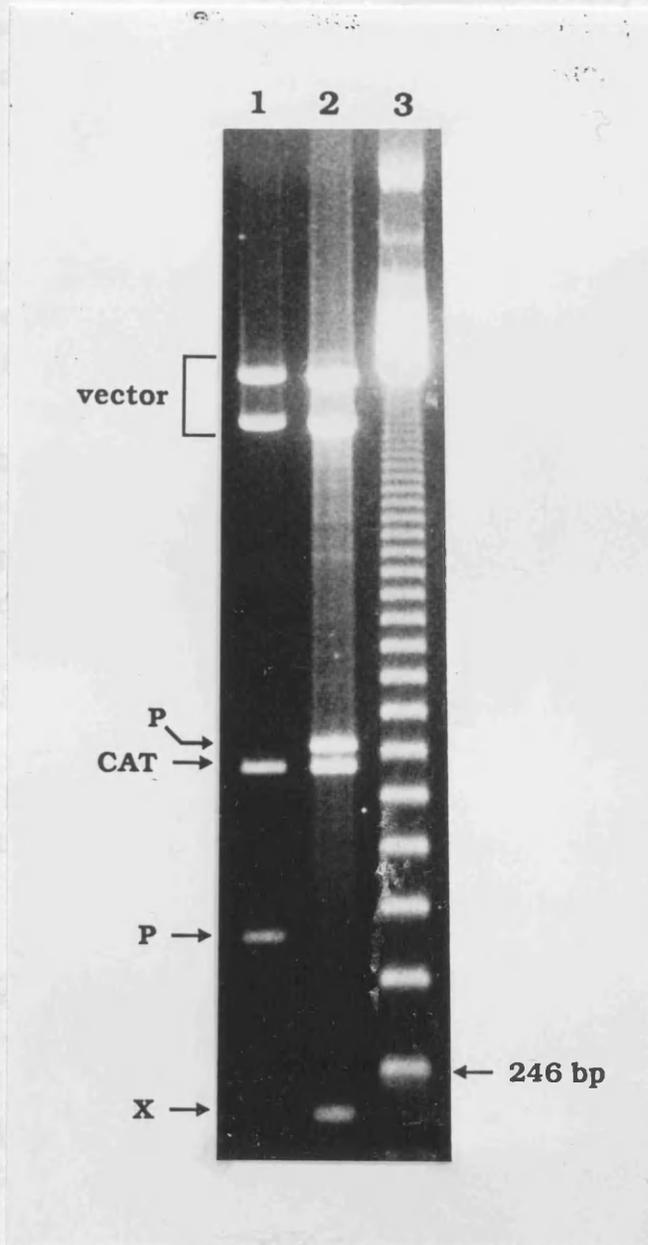
4.3. Infectivity

The cells trans investigated. Table 5.1 of column 6. The pNF1CAT and pNF2CAT and 25-fold 33 promoter, unlike the R promoter of alone exhibit it is medial separate.

4. ANALYSIS

4.1. Generation

The 3 were isolated (CAT) vector as described in section 3.1. These viruses, designated with the prefix 'NF' were used for further characterization of the NF promoter. In vivo infection of NIH 3T3 and 293T neuroblastoma cells.



indicated by the level of the other, and gly in the 6. The NF

and pNF2CAT to the 1992

the NF/CAT and LAT promoter/CAT fusion plasmids described in section 3C were all carried out at the same time.

The results demonstrate that both NFCAT clones perform well as promoters in this assay, exhibiting as least as much activity as pgDCAT. The activity exhibited by pNF2CAT is twice as much as pNF1CAT, thereby demonstrating that in BHK cells, sequences upstream of the *Bam*HI site at -296 of the NF sequence can contribute to NF promoter activity.

4C.3. Infection Studies

The effect on NF promoter activity of infecting cells transfected with the pNFCAT constructs was investigated. The results are shown in columns 4 and 5 of Table 8 together with the level of induction indicated in column 6. They show that the CAT activity exhibited by the pNF1CAT and pNF2CAT are induced to very high levels (132 and 95-fold respectively), thereby demonstrating that the NF promoter, like the HSV-1 lytic cycle promoters, and unlike the HSV-1 LAT promoter, responds strongly in the presence of HSV-1 *trans*-activating factors. As both NF clones exhibit this response, the sequences through which it is mediated are presumably located within the NF1 sequence.

4D. ANALYSIS OF THE NF PROMOTER IN THE VIRUS VECTOR

4D.1. Generation of NFCAT Viruses

The *Xba*I fragments from both pNF1CAT and pNF2CAT were isolated and purified, and introduced into the 1802 (LAT+) vector as described in section 3D.4. These viruses, designated with the prefix "v" were used for further characterisation of the NF promoter during lytic infection of BHK and C1300 neuroblastoma cell lines.

4D.2. Analysis of NFCAT Virus Genomes

Total DNA was prepared from BHK monolayers infected with vNF1CAT and vNF2CAT, and analysed by restriction enzyme digestion with *Xba*I and *Hind*III, running on a 1% agarose gel alongside the *Xba*I/*Hind*III digests of the corresponding NFCAT plasmids, followed by Southern transfer to nitrocellulose filters and probing with the *Xba*I/*Hind*III NF promoter fragment derived from pNF1CAT. Figure 35 shows that the viral DNAs yield NF promoter fragments of similar size to those yielded by the corresponding plasmids, thus indicating that the NF promoter fragments located within the virus genome are intact, and free from any rearrangements.

4D.3. CAT Expression from Recombinant Viruses

3.1. BHK Cells The level of CAT activity exhibited by vNF1CAT and vNF2CAT, together with controls vFJ5, vFJ7, and vFJ10 were determined at 6, 12, 24 and 48h pi at a moi of 5 at 37°C. A typical set of data presented on a linear plot, is shown of Figure 36(a). The data parallels that of the transfection studies by demonstrating that the NF promoter performs very strongly in BHK cells. The CAT activity exhibited by vNF2CAT is twice that of vNF1CAT thereby confirming that the upstream 5' flanking regions exert an influence on NF promoter activity in BHK cells.

3.2. C1300 Cells As for the BHK cells, the level of CAT activity exhibited by vNF1CAT, vNF2CAT and controls vFJ5, vFJ7 and vFJ10 were determined at 6, 12, 24 and 48h pi with a moi of 5 at 37°C. A typical set of data, again presented on a linear plot is shown on Figure 36(b). The data indicates that in neuroblastoma cells, the activity exhibited by vNF2CAT is greater than that exhibited by the lytic cycle promoter in vFJ7, and indeed is almost identical to that of vFJ10. The activity exhibited by vNF2CAT is five times that of vNF1CAT, thereby indicating

Figure 35. Southern blot analysis of NF promoter/reporter gene fusion viruses. Viral DNA was prepared from infected cells when extensive cpe became apparent. Each viral and corresponding plasmid DNA was digested with *Xba*I and *Hind*III and subjected to Southern blot analysis using a nick translated *Xba*I/*Hind*III NF promoter probe derived from pNF1CAT.

| | Band Size (bp) |
|------------------------------------|----------------|
| Track 1: vNF1CAT infected cell DNA | 380 |
| Track 2: pNF1CAT | |
| Track 3: vNF2CAT infected cell DNA | 980 |
| Track 4: pNF2CAT | |

The positions of the NF promoter fragments from each recombinant virus are indicated (P1 and P2).

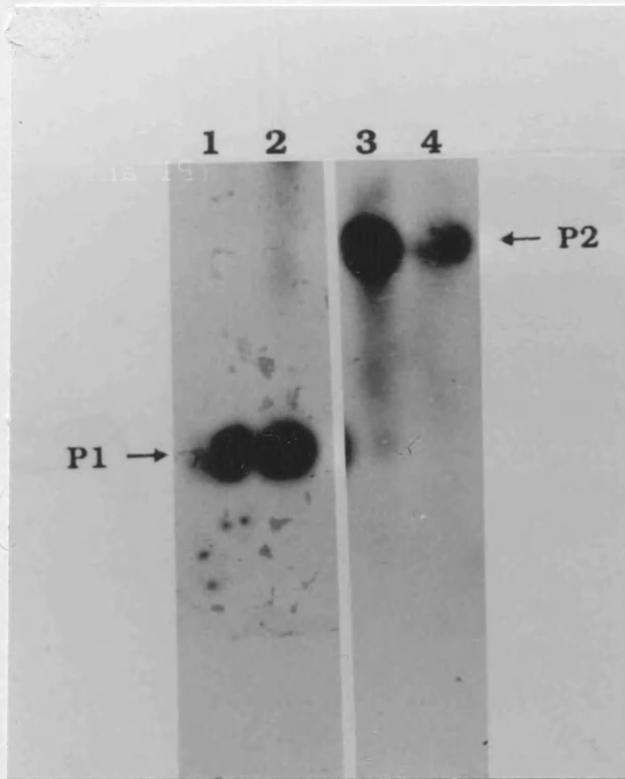
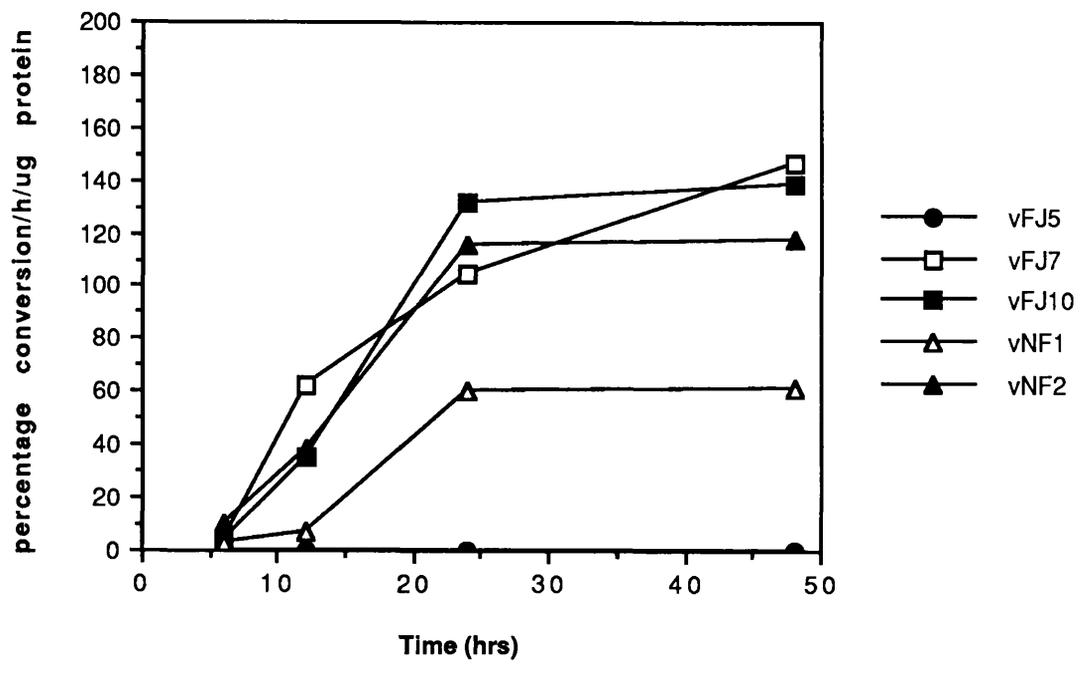
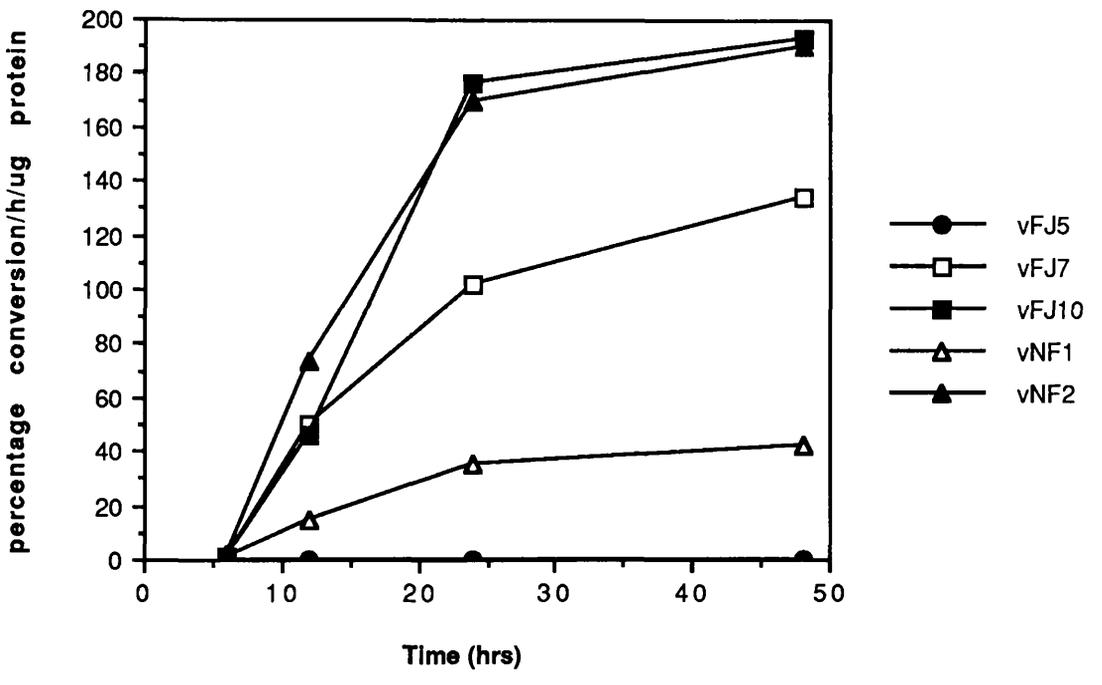


Figure 36. CAT activities produced by recombinant NF promoter viruses at various times after infection of BHK and neuroblastoma C1300 cells. a) CAT activities generated by vFJ5, vFJ7, vFJ10, vNF1CAT and vNF2CAT in BHK cells. b) CAT activities generated by vFJ5, vFJ7, vFJ10, vNF1CAT and vNF2CAT in neuroblastoma C1300 cells. CAT activities were determined at 6, 12, 24 and 48 h post infection and expressed as the percentage [¹⁴C]chloramphenicol converted per h per μ g protein. The symbols representing each virus are indicate in the legends beside each graph.

a) BHK cells



b) C1300 cells



that sequences upstream from -296 of the NF gene have greater effect on NF promoter activity in neuroblastoma cells than in fibroblastic cells. These differences were consistently reproduced in every experiment, and suggests that this upstream region of the NF gene may contain neuronal specific sequences capable of enhancing NF promoter activity in these cells.

4E. DISCUSSION

The main conclusions to be drawn from the transfection assays (section 4C) are that the two HNF-L promoter/CAT fusions (extending back to -296 and -896 from the cap site of the HNF-L gene) perform as functional promoters in BHK cells and exhibit at least as much activity as pgDCAT. pNF2CAT consistently exhibited twice as much activity as pNF1CAT, demonstrating that, at least in BHK cells, sequences upstream from -296 of the HNF-L sequence contribute to HNF-L promoter activity. Other studies, (Julien *et al.*, 1987a; Monteiro & Cleveland, 1989), have demonstrated that the HNF-L gene is transcribed from its own promoter and processed correctly in mouse L-cell and Hela cell lines, despite the fact that these, and presumably BHK cells, do not express their endogenous NF genes. In mouse L-cells, it was shown that the majority of NF-L proteins co-polymerised with the vimentin IF network (Monteiro & Cleveland, 1989).

Interestingly, it was found that upon infection with HSV-1, CAT activity exhibited by cells transfected with the HNF-L/CAT fusions was induced at least as much as lytic cycle promoters in pgDCAT and pFJ10. Since both NF clones exhibited this response, the sequences through which it is mediated are presumably contained within the NF1 clone (-296 to +84). While the effects of HSV-1 infection on cellular promoters has not been extensively studied, it

has been noted that although host transcription in general is reduced, a small number of cellular proteins are observed to increase in abundance, eg, heat-shock genes (Notarianni & Preston, 1982; Russell *et al.*, 1987a). Everett, (1984a, 1985) has shown that when the rabbit β - and ϵ -globin promoters are transfected into BHK cells, they were activated upon infection with HSV-1. It was also demonstrated that when the rabbit β -globin promoter is introduced into cell lines by biochemical transformation, it is stimulated by HSV-1 infection, whereas the endogenous β -globin promoter in rabbit kidney cells is not (Everett, 1985). It seems that the activity exhibited by the HNF-L promoter in BHK cells (and the β - and ϵ -globin promoters in transfection assays (Everett, 1984a, 1985)) probably reflects the relative chromatin structure of the endogenous and transfected genes. While the inactive endogenous β -globin gene in rabbit kidney cells is probably wrapped up in a densely packed chromatin structure, and therefore unavailable for activation, transfected plasmid DNA is assembled into an open nucleosomal structure accessible to nuclease digestion (Cereghini & Yaniv, 1984), and therefore presumably, the cellular transcription apparatus and HSV-1 transcription factors. The same is probably true for the NF-L promoter described here.

The high level of NF promoter activity exhibited in the infected transfection experiments is paralleled in the virus vector experiments detailing CAT activity during lytic infection of tissue culture cells. Expression from the HNF-L promoter in C1300 neuroblastoma cells, which have been shown to express their endogenous NF genes (cited in Julien & Grosveld, 1991) demonstrated that the CAT activity exhibited by the strongest HNF-L promoter clone (vNF2CAT) was no greater than that exhibited by the HSV-2 IE4/5 promoter in vFJ10. This is perhaps a reflection of these promoters working at maximum capacity in the context of the lytic infection, and it would be interesting to see how

these promoters would perform relative to each other in transfection assays in C1300 cells. In another study, the rabbit β -globin promoter, when introduced into the HSV genome, has been shown to be regulated as an early viral gene during infection of fibroblasts (Smiley *et al.*, 1987). It was also noted here that while the activity exhibited by vNF2CAT is twice that of vNF1CAT in BHK cells, it is almost five times greater in C1300 cells, thereby suggesting that sequences upstream of -296 may contribute to neuronal specificity of the HNF-L gene.

Studies of HNF-L gene expression in transgenic mice (Julien *et al.*, 1987b) have found that a 21kb fragment, including 14kb of 5' flanking sequences, specifically expressed the HNF-L transgene in neuronal tissues. It was subsequently found that a transgenic carrying the HNF-L gene and 292bp of 5' flanking sequence expresses the transgene specifically in the nervous system, while a deletion to -55 abolishes transcription (cited in Julien & Grosveld, 1991). Another study (Vidal *et al.*, 1990), has shown that when the thy-1 promoter is replaced with 2.5kb of flanking sequence of the HNF-L promoter in a thy-1 transgene (a member of the immunoglobulin supergene family expressed specifically in neurons), the thy-1 hybrid is still expressed in a neuronal specific manner, but retains the thy-1 developmental expression pattern, thus demonstrating that the NF promoter alone is not sufficient for the early developmental expression normally associated with the NF gene. It is therefore quite likely that some regulatory signals of the NF gene, like those of the thy-1 gene (Vidal *et al.*, 1990) are located downstream of the promoter region.

The transgenic experiments described above would appear to suggest that neuronal specific expression can be achieved with both the NF1 and NF2 promoter fragments. It would therefore be interesting to examine whether this is the case when fused to a reporter gene in the context of

the latent HSV-1 genome. Unfortunately, the NFCAT viruses are unsuitable for this kind of study since the CAT assay is probably not sensitive enough to allow detection of the limited amount of HSV gene expression in latently infected ganglia. The virus vector system has been adapted to use the *lacZ* gene instead (see Chapter 5) thereby allowing the use of histochemical assays to examine expression of the promoter/*lacZ* gene fusions in individual cells. These viruses were in the process of being made at the time of this study, and it will be of great interest to see what they tell us about gene expression from the HSV-1 genome during latency.

If the NF-L promoter did prove to be active in the context of the latent virus genome, it should be possible to clone the whole human NF-L gene (including introns) together with the 296bp promoter flanking sequence as a 4.5kb fragment into the 1802 vector genome. Human NF-L protein expression from the latent HSV genome in murine DRG neurons could be distinguished from the endogenous NF-L proteins by use of a monoclonal antibody which detects the human, but not the mouse NF-L proteins (Julien & Grosveld, 1991). Expression of NF and other neuronal specific genes from latent HSV-1 genomes in the murine nervous system should provide an alternative to the use of transgenic mice for the study of tissue specific gene expression, and interaction of NF proteins with the cytoskeleton. If the NF promoter did not prove to be active, then the LAT or MMTV LTR promoters which are functional during latency, could be used as alternatives to drive heterologous gene expression from the latent virus genome.

CHAPTER 5: ANIMAL LATENCY STUDIES

5A. Introduction

The ultimate aim of this study was to examine gene expression from the NF and LAT promoter fragments in the context of the HSV-1 genome during latency. The NFCAT and LATCAT viruses are, however, unsuitable for this work, since the CAT assay is unlikely to be sensitive enough to allow detection of HSV gene expression in latently infected ganglia. For this reason, it was decided to substitute the *lacZ* gene for the CAT gene, thus allowing use of histochemical assays for detection of gene expression in individual cells in latently infected ganglia. These viruses were in the process of being made at the time of this study, however, one virus, vFJ12 (containing the HSV-2 IE-4/5 promoter fused to the *lacZ* reporter gene) was available, and was initially tested in the animal latency system.

The mouse footpad model was used, whereby virus is inoculated into the right rear footpad, and following establishment of latency, typically after 4 weeks, dorsal root ganglia are dissected and either explanted to test for virus reactivation, or further processed and analysed for characteristics of virus gene expression. The first part of this study was to determine that the virus vector can establish latency, and that the 1802 vector expresses LATs.

5B. Latency Characteristics of the Virus Vector

Comprehensive explantation reactivation and pathogenesis studies of the 1802/1804 vectors were not carried out, however, some interesting observations of these characteristics were made. It was noted that vNF1CAT, vFJ12 (see Section 5D.2) and indeed, the other insertion-containing recombinants, were non-pathogenic in the footpad model; inoculation of up to 2×10^8 pfu (the maximum available dose) failed to register any ill effect. This

contrasts with HSV-1 17+ which has a lethal dose of 5×10^5 pfu, and interestingly, 1804 where 2/3 mice died after inoculation with a dose of 3×10^7 pfu. This suggests that insertion of sequences into the vector affects pathogenicity of the virus. Rixon & Mclauchlan (1990), observed that the amount of a $M_r 21,000$ (21K) protein synthesised by vFJ7 and vFJ10 is reduced compared with the parental 1802 vector. The 21K is encoded by US11, one of the genes flanking the unique *Xba*I site in 1802, and it was proposed that this reduction could be a result of read through transcription from the inserted sequences in vFJ7 and vFJ10. Other studies (Meignier *et al.*, 1988; M.Y.M Taha, Ph.D. Thesis, University of Glasgow, 1990) have implicated a role for US11 in pathogenesis of HSV-1, and it is intriguing to consider that the non-pathogenicity of the insertion-containing virus vector is due to the reduction of 21K expression. Further characterisation of the 1802/1804 vectors is obviously necessary to determine whether this is the case.

In reactivation/explantation experiments, reactivation of vNF1CAT was consistently observed, typically from 2-3 ganglia 8-10 days post explant, thereby demonstrating that by this criterion, vNF1CAT is able to establish a reactivatable latent infection in the footpad model.

5C. HSV 1802 Expresses LAT

In order to confirm that HSV-1 1802 expresses LAT, *in situ* hybridisation analysis was performed on sections of latently infected ganglia from mice that had been inoculated with 2×10^8 pfu of vNF1CAT (an 1802-derivative). These were probed with ^{35}S -labelled riboprobes derived from a 219bp *Sa*II/*Eco*RI fragment excised from plasmid p110E35 (Everett, 1987), and ligated into *Sa*II/*Eco*RI digested pT7T3 19U such that riboprobes synthesised from the T7 promoter were antisense to the IE-1 mRNA, and those from the T3

Figure 37. *In situ* hybridisation riboprobes. The top line gives a schematic representation of the region of TR_L/IR_L between the 5' ends of the 2 kb LAT and the 5' end of the IE-1 gene. The third line down gives a partial restriction enzyme profile of this region, indicating the positions of unique *Sal*I and *Eco*RI sites. The latter is derived from an *Eco*RI linker insertion IE-1 mutant (p110E35; Everett, 1987). The *Sal*I/*Eco*RI fragment from p110E35 was excised and ligated into *Sal*I/*Eco*RI digested pT7T3 (shown below) such that riboprobes synthesised from the T7 promoter are antisense to the IE-1 mRNA, and those from the T3 promoter antisense to LAT.

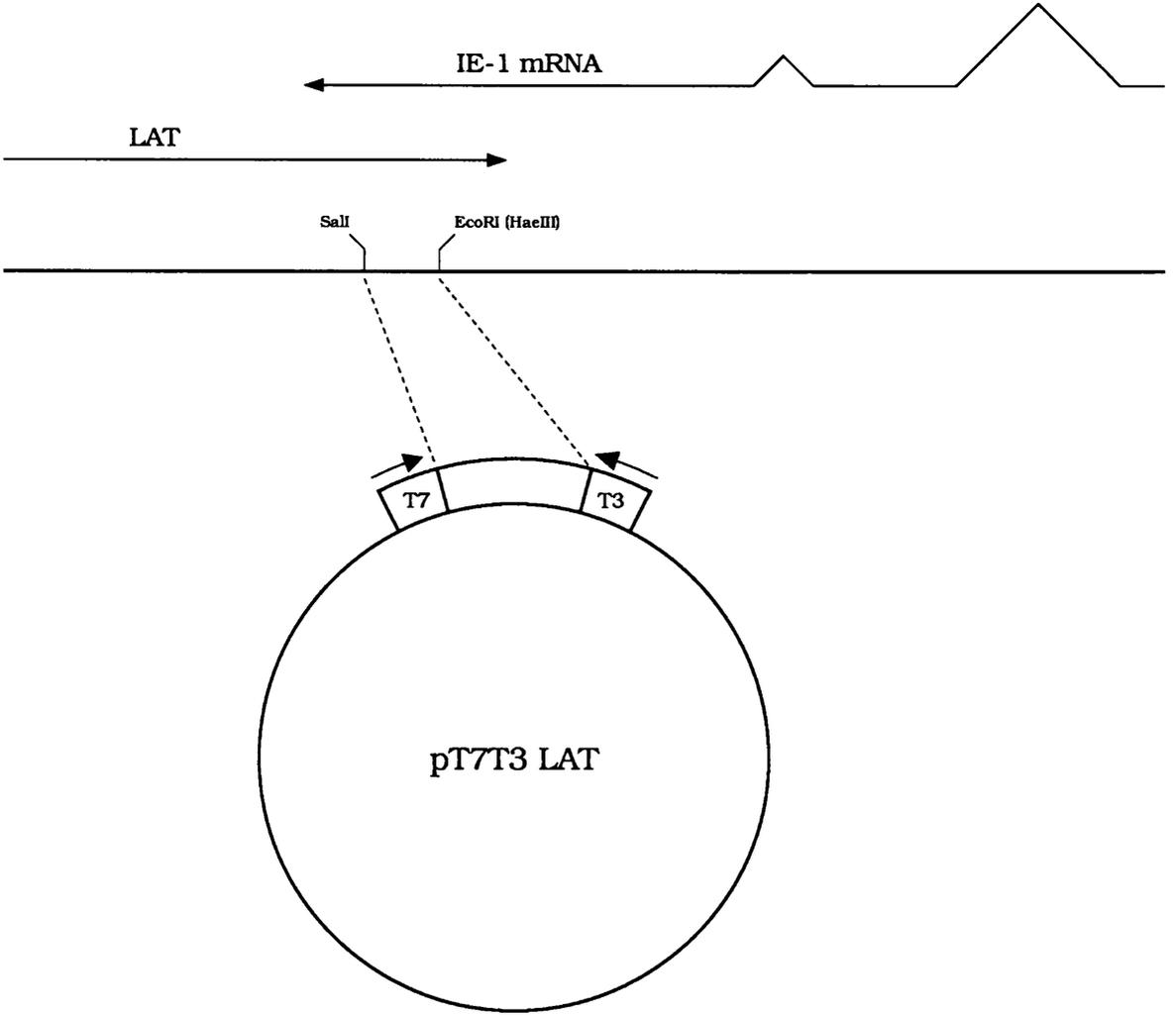
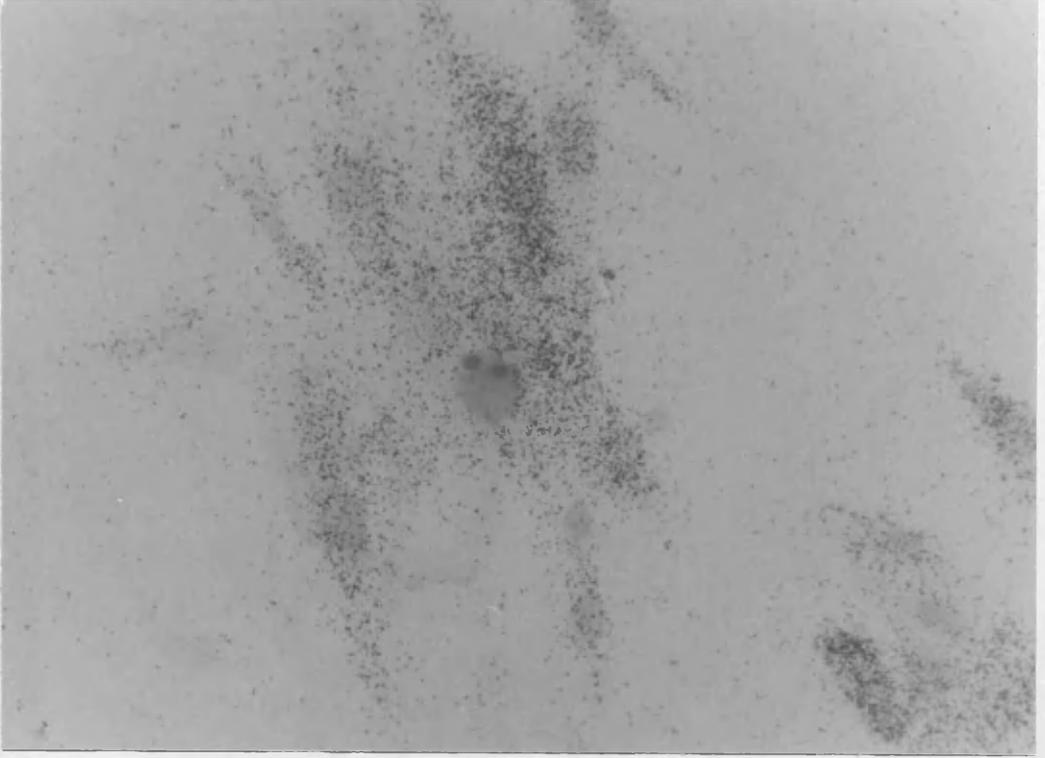
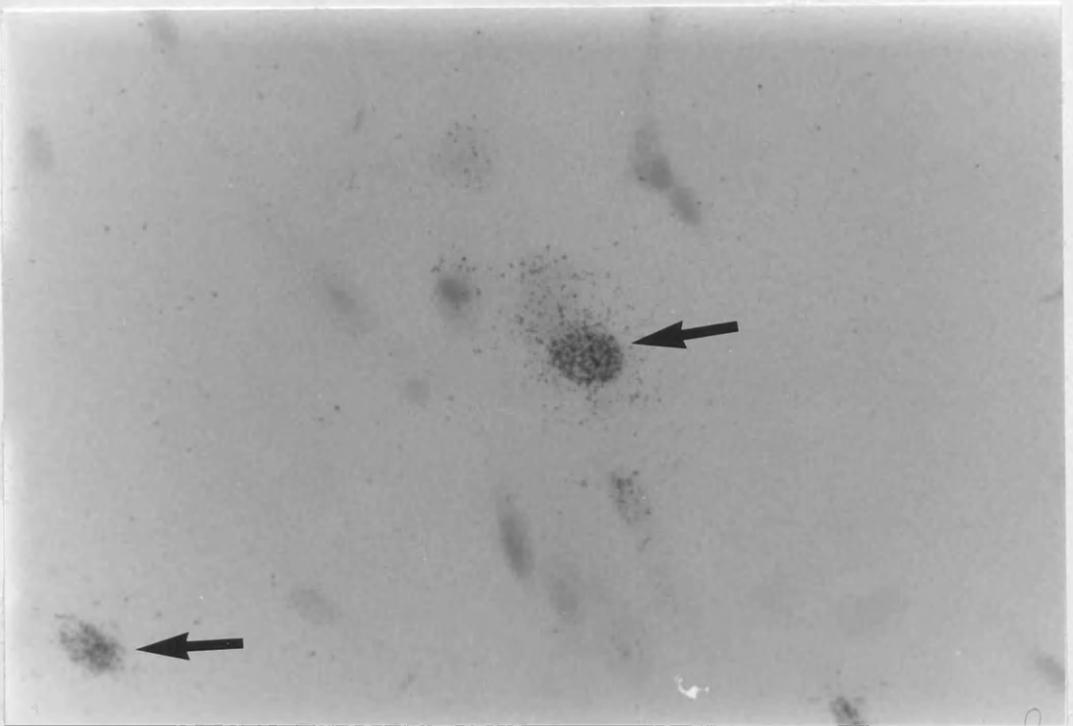


Figure 38. *In situ* hybridisation of lytically infected BHK cells using ³⁵S-labelled riboprobes. a) Lytically infected BHK cells hybridised with an anti-IE-1 riboprobe. Labelling is predominantly cytoplasmic. b) Lytically infected BHK cells hybridised with an anti-LAT riboprobe. The nuclei of two cells (marked with an arrow) demonstrate strong signals restricted largely to the nucleus.

a



b



20µm

Figure 39. *In situ* hybridisation of sections of latently infected murine spinal ganglia using ³⁵S-labelled riboprobes. The spinal ganglion section was probed with an antisense probe to LAT, and stained with haematoxylin and eosin which stains the nuclei and cytoplasm of neuronal cells, but only the nuclei of non-neuronal cells. A single neuron (marked with an arrow) demonstrates strong signals restricted to the nucleus.

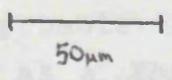
promoter antisense to LAT (see Figure 37). These riboprobes were also used on coverslips of BHK cells 24h pi with HSV-1 17 at a mol. of 0.01. This work was carried out in conjunction with Dr. Marion Pugh-Preece, Southern General Hospital, Glasgow.

Figure 38(a) shows HSV-1 infected BHK cells probed with the patterns observed in Figure 38(b)). consisted of transcribed also observed in ganglia. shows an counterstain localised the pattern of. 1987. VNFICAT. expressed. SP. Actin. SV40 promoter (Bixon & IE-4/5 promoter excised vector. the CNT blue/white recombinant



Figure 38(b) shows HSV-1 infected BHK cells probed with the patterns observed in Figure 38(b)). consisted of transcribed also observed in ganglia. shows an counterstain localised the pattern of. 1987. VNFICAT. expressed. SP. Actin. SV40 promoter (Bixon & IE-4/5 promoter excised vector. the CNT blue/white recombinant

SP.1. Construction of the IE-4/5 Promoter/Lat Plasmid
 The HSV-1 IE-4/5 promoter sequence in plasmid pE319 was excised and purified as a λ DNA/BamHI fragment. This



promoter antisense to LAT (see Figure 37). These riboprobes were also used on coverslips of BHK cells 24h pi with HSV-1 17+ at a moi of 0.01. This work was carried out in conjunction with Dr. Marion Ecob-Prince, Southern General Hospital, Glasgow.

Figure 38(a) shows HSV-1 infected BHK cells probed with the anti-IE-1 riboprobe, and as expected, dense patterns of cytoplasmic and nuclear graining can be observed in infected cells. With the anti-LAT probe (Figure 38(b)), graining is observed almost exclusively in nuclei, consistent with the known localisation of the LAT transcripts in latently infected ganglia. This pattern is also observed when the sections of latently infected ganglia are probed with the anti-LAT riboprobe; Figure 39 shows an extremely dense area of graining which, when counterstained with haematoxylin and eosin, is found to be localised to the nucleus of a neuron. This is identical to the pattern of LAT expression shown by others (Stevens *et al.*, 1987), and is therefore a clear demonstration that vNF1CAT, and thus presumably the parental 1802 vector, expresses LAT.

5D. Activity of the HSV-2 IE-4/5 Promoter during Latency

The strategy for this study entailed replacing the SV40 promoter/enhancer driving *lacZ* gene expression in pFJ1 (Rixon & Mclauchlan, 1990; see Figure 40), with the HSV-2 IE-4/5 promoter. The IE-4/5 promoter/*lacZ* fusion could then be excised as an *Xba*I fragment and ligated into the 1802 vector. The high IE-4/5 promoter activity, as observed from the CAT assays in BHK cells (section 4C) would allow blue/white selection to be used when plaque purifying the recombinant virus.

5D.1. Construction of the IE-4/5 Promoter/*lacZ* Plasmid

The HSV-2 IE-4/5 promoter sequence in plasmid pFJ10 was excised and purified as a *Xba*I/*Hind*III fragment. This

was ligated into *Xba*I/*Hind*III digested pFJ1 (with the SV40 promoter/enhancer removed). The *Bam*HI site downstream of the *lacZ* gene was converted to a *Xba*I site by *Bam*HI digestion, blunt-ending with T4 DNA polymerase, and ligation of the phosphorylated linker 5'-CTCTAGAG-3' thereby generating plasmid pFJ10Gal+*Xba*I (this treatment regenerated *Bam*HI sites on either side of the *Xba*I linker). A diagnostic restriction profile of this plasmid is shown on Figure 41.

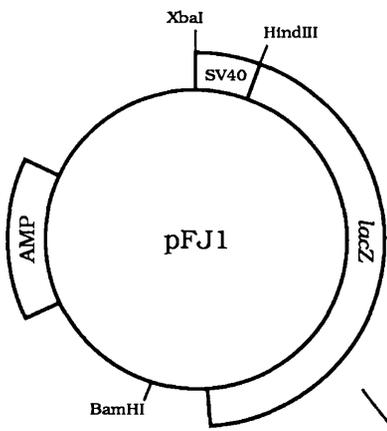
5D.2. Construction of vFJ12

The *Xba*I fragment from pFJ10Gal+*Xba*I was isolated and purified, and introduced into the 1802 vector as described in section 3D.4. This virus, designated vFJ12, was used for measuring activity of the HSV-2 IE-4/5 promoter during HSV-1 latency.

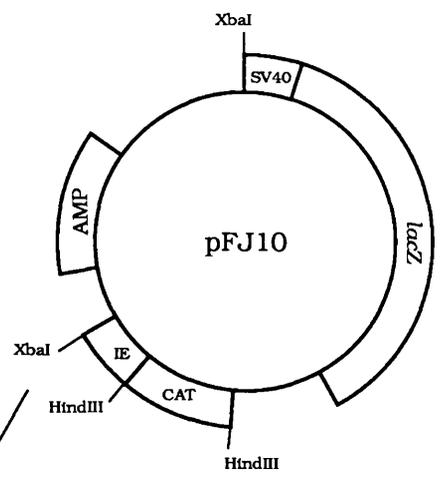
5D.3. Analysis of the vFJ12 Genome

Total cellular DNA was prepared from BHK monolayers infected with vFJ12 and analysed by restriction digestion with *Hind*III and *Xba*I. This analysis, however, failed to release the expected IE-4/5 promoter fragment (data not shown). Further analysis of restriction profiles of the viral DNA revealed that some form of genomic rearrangement had occurred within the U_L region. Figure 42 shows a restriction map of this region of the HSV-1 genome, and Figure 43 depicts a series of restriction profiles of vFJ12 and HSV-1 17+ DNA. The *Bam*HI profile of vFJ12 shows that both *Bam*HI *x* and *z* fragments are missing, and the appearance of an additional band marked X1. A densitometric scan of the negative for the photograph in Figure 43 also indicates that one copy of the *Bam*HI *y* band is missing as well (see Figure 44). The absence of the *Bam*HI *z* track is expected as the unique *Xba*I site utilized by the vector system is located there (Rixon & McLauchlan, 1990), and any insertion at this site will therefore cause a shift in the

Figure 40. Construction of pFJ10Gal+Xba. The *XbaI/HindIII* HSV-2 IE-4/5 promoter fragment was excised, purified and ligated into *XbaI/HindIII* digested and purified pFJ1 DNA to give pFJ10Gal. The unique *BamHI* site in pFJ10Gal was then converted to a *XbaI* site by *BamHI* digestion, 'blunt-ending' with T4 DNA polymerase, and ligation of a *XbaI* linker. This treatment regenerated *BamHI* sites on either side of the *XbaI* linker insert. SV40; SV40 early promoter. *lacZ*; *E.coli lacZ* gene. CAT; chloramphenicol acetyltransferase gene. AMP; ampicillin resistance gene.

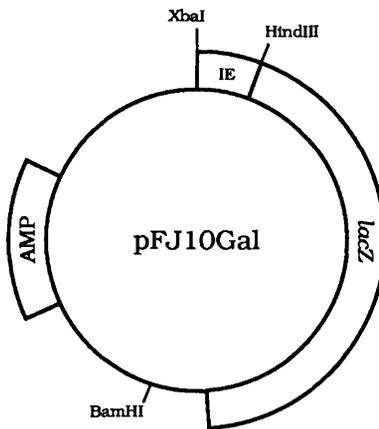


Purify XbaI/HindIII digested vector



Excise and purify XbaI/HindIII IE-4/5 promoter fragment

Ligate



Insert XbaI linker into BamHI site

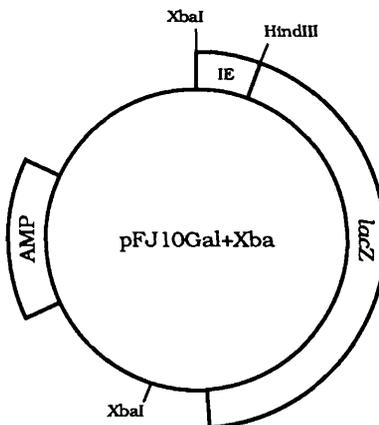


Figure 41. Restriction digest demonstrating integrity of pFJ10Gal+Xba. This Figure shows a *XbaI/HindIII* digest of pFJ10Gal+Xba DNA (lane 2) run alongside a 123bp ladder (lane 1) on a 1% agarose gel stained with EtBr. This digest releases a *XbaI/HindIII* IE-4/5 promoter fragment, which from the gel, runs alongside the 492bp ladder fragment. This is consistent with the expected size of 470bp.

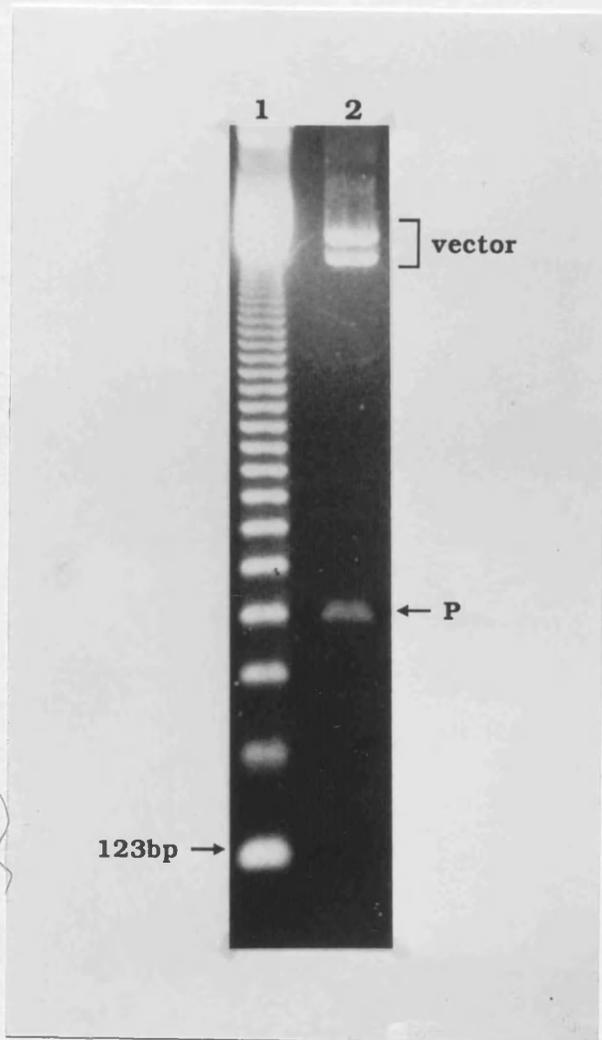


Figure 42. Map of the S region of the HSV-1 genome. The top line shows the scale of the figure in map units. The second top and bottom lines show the position and scale of the figure in kbp. The third and fourth top lines show *Bam*HI and *Hind*III restriction maps of the S region respectively. The U_S region, flanked by the repeat regions, TR_S and IR_S are represented by the open boxes in the centre of the diagram. The horizontal arrows above and below the open boxes represent RNAs; the arrows give the direction of these transcripts. The shaded black boxes represent the ORF's of IE genes; boxes with horizontal lines represent early genes; boxes with diagonal lines represent early late (delayed early) genes, and cross-hatched boxes represent late genes. Open boxes directly below the S region (labelled R) represent tandem repeats. The position of the unique *Xba*I site utilized in the 1802 and 1804 vectors is also indicated. This Figure is reproduced and adapted from one originally published in McGeoch *et al.* (1990), with permission.

Figure 43. Restriction profiles of vFJ12 and HSV-1 17+ DNA. Lanes 1 and 2 shows *Bam*HI restriction profiles of vFJ12 and HSV-1 17+ virion DNA. The HSV-1 17+ bands are labelled from o to d'. As observed from lane 2, *Bam*HI x and z are missing from the vFJ12 profile which also has an additional band, marked X1, present. Lanes 4 to 7 show additional *Bam*HI and *Bam*HI/*Hind*III profiles of vFJ12 and HSV-1 17+ electrophoresed further than those in lanes 1 and 2. Lane 3 shows a 1kb DNA ladder with bands labelled 2 (2kb) to 8 (8kb). The fragments of the *Bam*HI profile of HSV-1 17+ (lane 7) are labelled l to z. The *Bam*HI/*Hind*III profile of vFJ12 has an additional band marked X2.

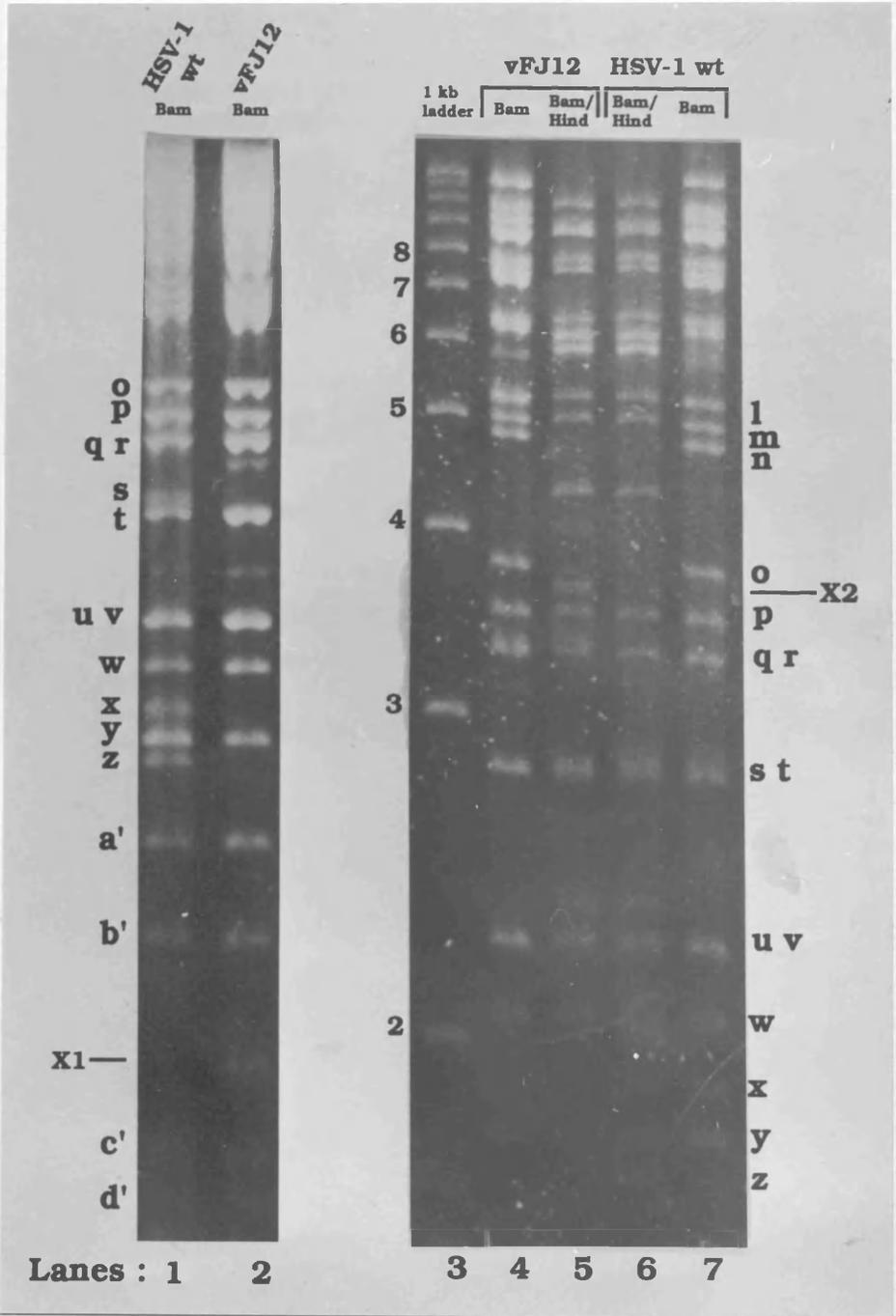
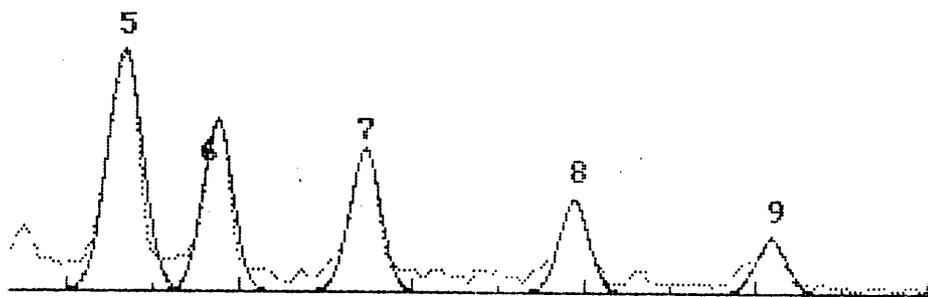


Figure 44. Densitometric scans of vFJ12 and HSV-1 wt restriction profiles. A negative of the photograph showing the *Bam*HI restriction profiles of vFJ12 and HSV-1 wt in Figure 43 was scanned using a Hoefer Scientific Instruments GS300 transmittance/reflectance densitometer. The vFJ12 profile is shown on the upper scan while HSV-1 wt is on the lower. Both scans show the region between *Bam*HI *u* and *v*, (corresponding to peak 5) and *Bam*HI *b'* (corresponding to peak 9 on vFJ12 and peak 11 on HSV-1 wt.)

| | |
|--|---|
| vFJ12: Peak 5: <i>Bam</i> HI <i>u.v.</i> | HSV-1 wt: Peak 5: <i>Bam</i> HI <i>u,v.</i> |
| 6: <i>Bam</i> HI <i>w.</i> | 6: <i>Bam</i> HI <i>w.</i> |
| 7: <i>Bam</i> HI <i>y.</i> | 7: <i>Bam</i> HI <i>x.</i> |
| 8: <i>Bam</i> HI <i>a'.</i> | 8: <i>Bam</i> HI <i>y.</i> |
| 9: <i>Bam</i> HI <i>b'.</i> | 9: <i>Bam</i> HI <i>z.</i> |
| | 10: <i>Bam</i> HI <i>a'.</i> |
| | 11: <i>Bam</i> HI <i>b'.</i> |

The *Bam*HI *y* peak on the wt profile is larger than those of it's neighbours indicating that it is present at a higher molar ratio, ie, present as two copies. In contrast, the *Bam*HI *y* peak on the vFJ12 profile is comparatively smaller, especially when compared to the *Bam*HI *w* peak. This indicates that *Bam*HI *y* is present as a single copy in vFJ12.

vFJ12



HSV-1 wt

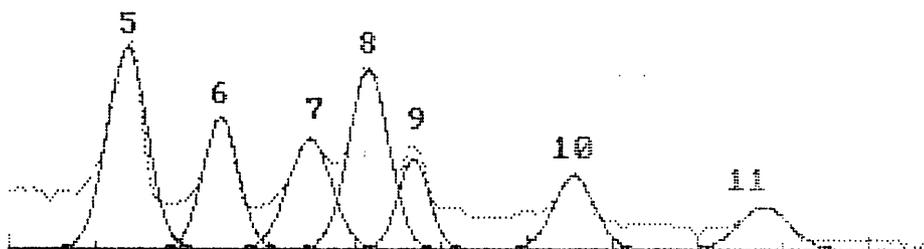


Figure 45. DNA sequence of the region upstream from the *lacZ* gene in pFJ10Gal and vFJ12. The first four tracks show the sequence of the HSV-2 IE-4/5 promoter upstream from the *lacZ* gene in plasmid pFJ10Gal. The sequencing reaction was carried out using a primer complementary to the 5' end of the *lacZ* gene. The second four tracks show a similar sequencing reaction carried out on vFJ12 virion DNA. Although the resolution of the vFJ12 sequence is very poor, close examination reveals certain similarities between the vFJ12 and pFJ10Gal tracks, thus confirming that at least a major portion of the HSV-2 IE-4/5 promoter remains upstream from the *lacZ* gene in vFJ12.

mobility of this band. The additional band marked X1 is, in fact, the half of *Bam*HI z between the *Bam*HI site at the 3' end of the *lacZ* gene (regenerated by the insertion of the *Xba*I linker into the *Bam*HI site of pFJ10Gal; see section 5D.1), and *Bam*HI j (see Figure 42). The size of this band (1135 bp) between *Bam*HI b' (1313 bp) and c' (428 bp) is indeed indicative of the orientation of the *lacZ* gene, ie, pointing away from the adjacent short repeat (Figure 42). The only significant difference between the *Bam*HI/*Hind*III profiles of vFJ12 and wt DNA (Figure 43) is an additional band marked 2X (running between *Bam*HI o (3866bp) and *Bam*HI p (3579bp). This is, in fact, a 3731bp band containing the *lacZ* gene between the *Bam*HI site at it's 3' end (re-created by ligation of the *Xba*I linker into the blunt-ended *Bam*HI site of pFJ10Gal), and the *Hind*III site between the IE-4/5 promoter and the *lacZ* ORF. In an attempt to determine the sequence immediately upstream of the *lacZ* gene, sequencing of purified vFJ12 virion DNA using a primer complementary to the 5' end of the *lacZ* was performed, and run on a gel alongside a similar sequencing reaction performed on pFJ10Gal DNA. Although the resolution of the vFJ12 sequencing tracks was poor, (Figure 45), it is quite clear that the pattern of bands in the viral DNA tracks are similar to those in the plasmid DNA tracks, thereby demonstrating that at least a major portion of the IE-4/5 promoter is still present upstream of the *lacZ* gene. At this point, the work for this thesis came to an end, and although the analysis of the genomic structure of this virus was being continued, latency studies had already been initiated in mice, and gave the interesting results described below.

5D.4. β -Galactosidase Expression during Acute and Latent Infection of Murine Sensory Ganglia

4.1. Expression during Acute Infection of Sensory Ganglia

To examine *lacZ* gene expression by vFJ12 during the lytic phase of viral infection, murine dorsal root ganglia were examined at 2, 4, 6, and 8 days following footpad inoculation with 1.6×10^7 pfu of vFJ12. β -galactosidase activity was detected by histochemical staining at all timepoints (except day 2) in the lumbar dorsal root ganglia of infected mice, and was visible in both whole mount preparations and wax-embedded sections. The majority were located in the L4 and L5 ganglia (see Table 9). The labelling observed in neurons on days 4 and 6 was so intense that in whole mounts, it could also be observed in axons, an effect probably due to diffusion of β -galactosidase from the neuronal soma (see Figure 46). These cells appeared completely opaque in sections of these ganglia (see Figure 47). Such labelling is possibly indicative of the very strong promoter activity associated with IE promoters during the acute phase of virus infection in ganglia which peaks at around 5 days post infection (see Section 1D.2). The histochemical staining of neurons at day 8 (Figure 47) was much less intense, and cytological detail could be observed in those cells. This lower level of β -galactosidase activity probably corresponds to a reduction in IE promoter activity following the peak of acute infection.

4.2. Expression during Latent Infection of Sensory Ganglia

To examine *lacZ* gene expression by vFJ12 during latency, whole mounts and sections of murine dorsal root ganglia were examined 1 month following footpad inoculation with up to 1×10^8 pfu of vFJ12. As observed from the whole mounts (Figure 48), and sections (Figure 49), histochemically stained neurons were observed in the spinal ganglia of these mice, but not those of uninfected controls, or indeed, controls infected with the parental vector virus. Labelled neurons were observed in both whole mounts and sections, and as during acute infection, most

| Ganglion | Number of blue cells per ganglion observed at: | | | |
|----------|--|--------|--------|----------|
| | 4 Days | 6 Days | 8 Days | 20 Weeks |
| L1 | 0 | 0 | 7 | 0 |
| L2 | 0 | 0 | 5 | 0 |
| L3 | 2 | 3 | 6 | 2 |
| L4 | 5 | 5 | 36+ | 17 |
| L5 | 1 | 8 | 28+ | 23 |

Table 9: Number of neurons expressing β -galactosidase in lumbar sensory ganglia dissected from the right hand side of mice examined at intervals following inoculation with vFJ12. One mouse was examined per time point except at 20 weeks when two mice were examined.

Figure 46. β -galactosidase activity in a whole mount of a dorsal root ganglion from a mouse acutely infected with vFJ12. At 4 days after inoculation, animals were sacrificed and spinal ganglia removed. This whole mount spinal ganglion was stained histochemically for β -galactosidase activity. The photograph clearly shows a densely stained neuron and associated axon.



150 μm

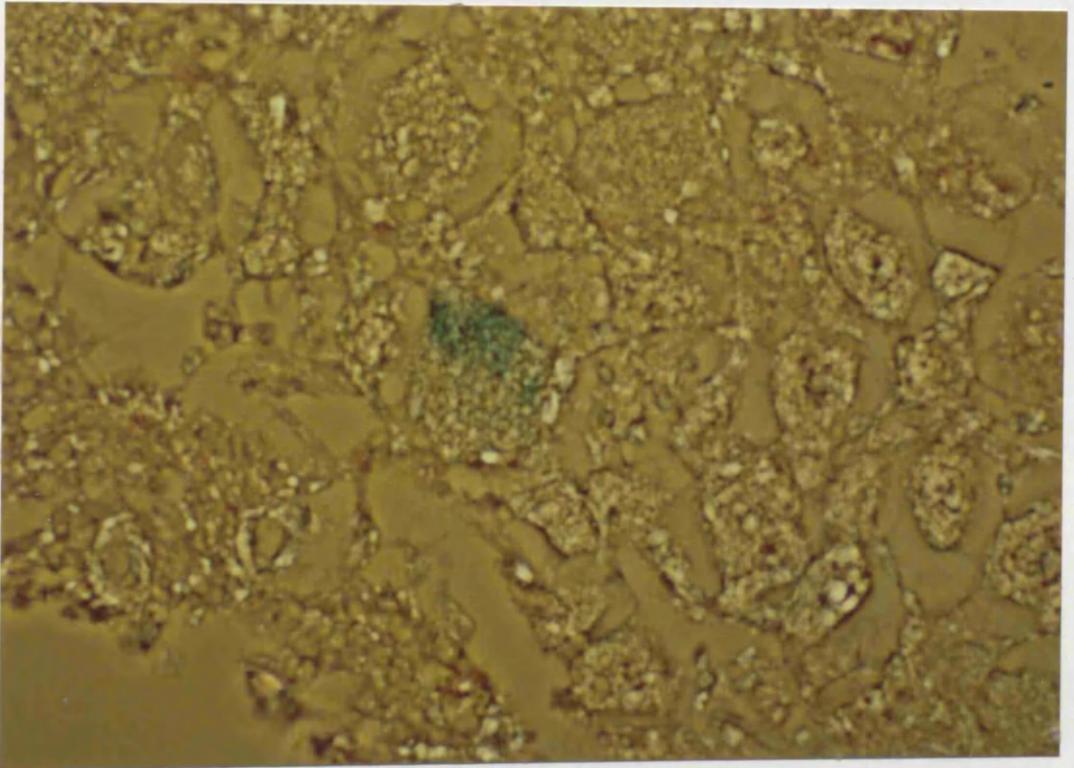
Figure 47. β -galactosidase expression in sections of dorsal root ganglia from mice acutely infected with vFJ12. At 4 and 8 days after inoculation, animals were sacrificed and spinal ganglia removed. Whole mounts were stained histochemically for β -galactosidase activity and sectioned at 6 μ m. a) shows a densely stained neuron in a section from an acutely infected ganglion at 4 days after inoculation. b) shows a lightly stained neuron in a section from an infected ganglion at 8 days after inoculation.

a



100µm

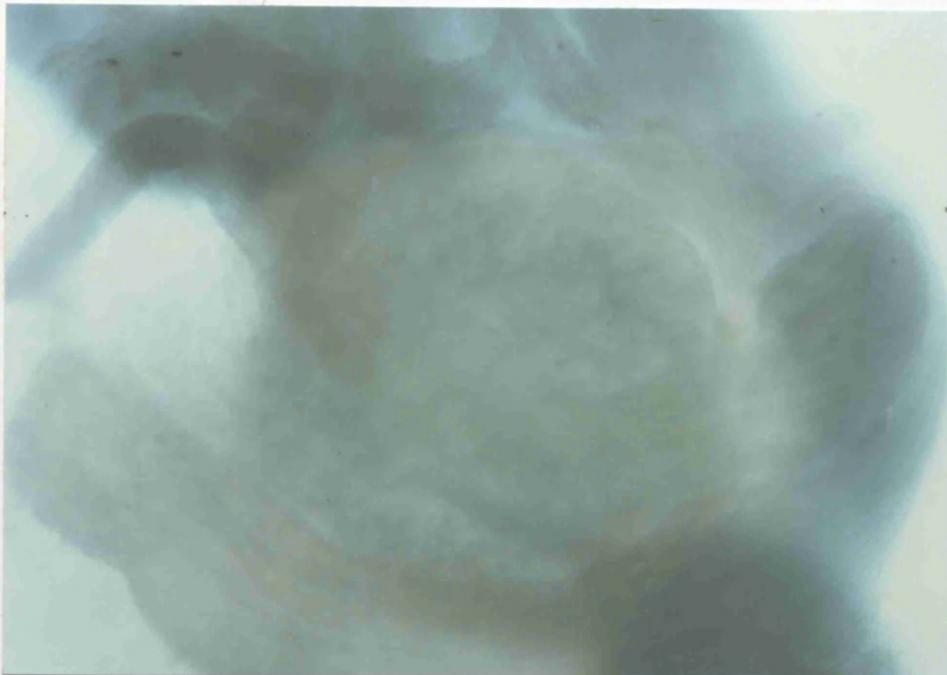
b



50µm

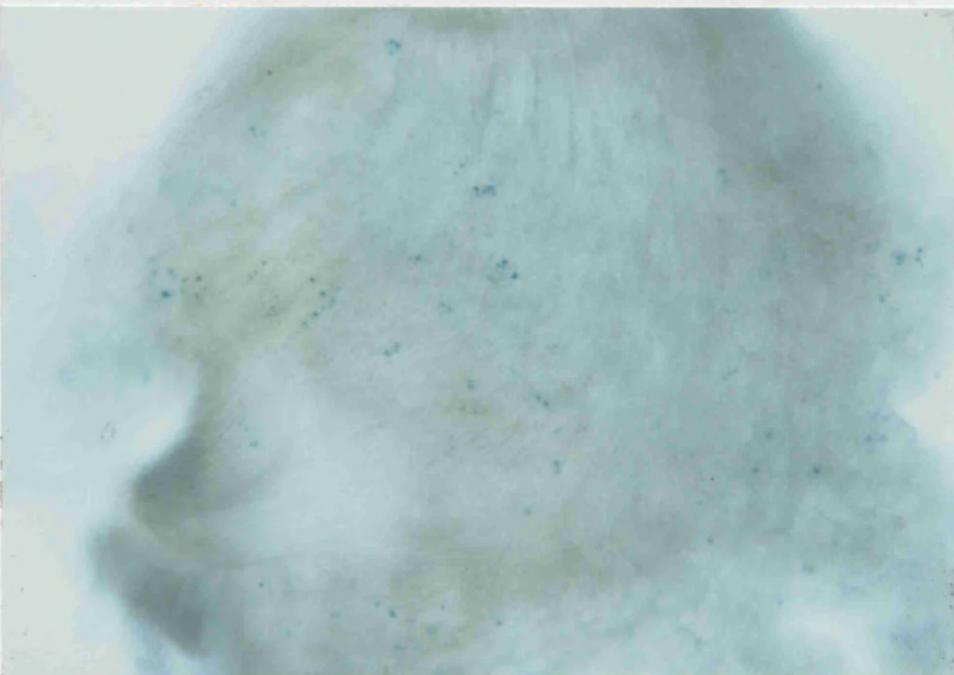
Figure 48. β -galactosidase expression in whole mounts from dorsal root ganglia from mice latently infected with vFJ12. At 6 months after inoculation, animals were sacrificed and spinal ganglia were removed. Whole mounts of spinal ganglia were stained histochemically for β -galactosidase activity. a) shows a control ganglion from an uninfected mouse. b) shows a ganglion from a mouse latently infected with vFJ12. c) shows a latently infected ganglion at higher magnification and demonstrates a speckled pattern of labelling within positive cells.

a



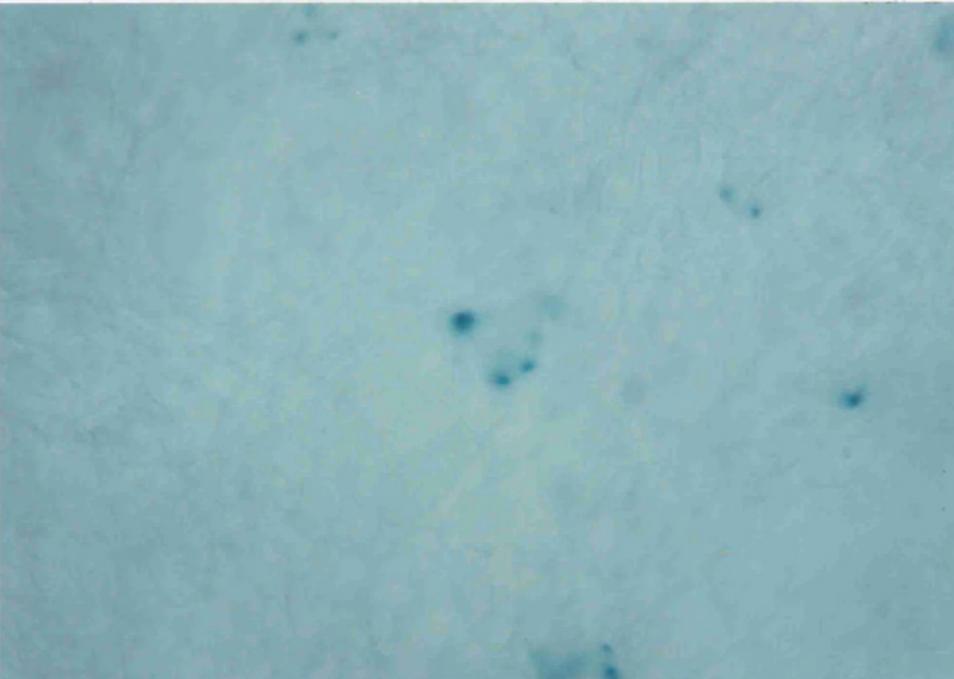
260µm

b



260µm

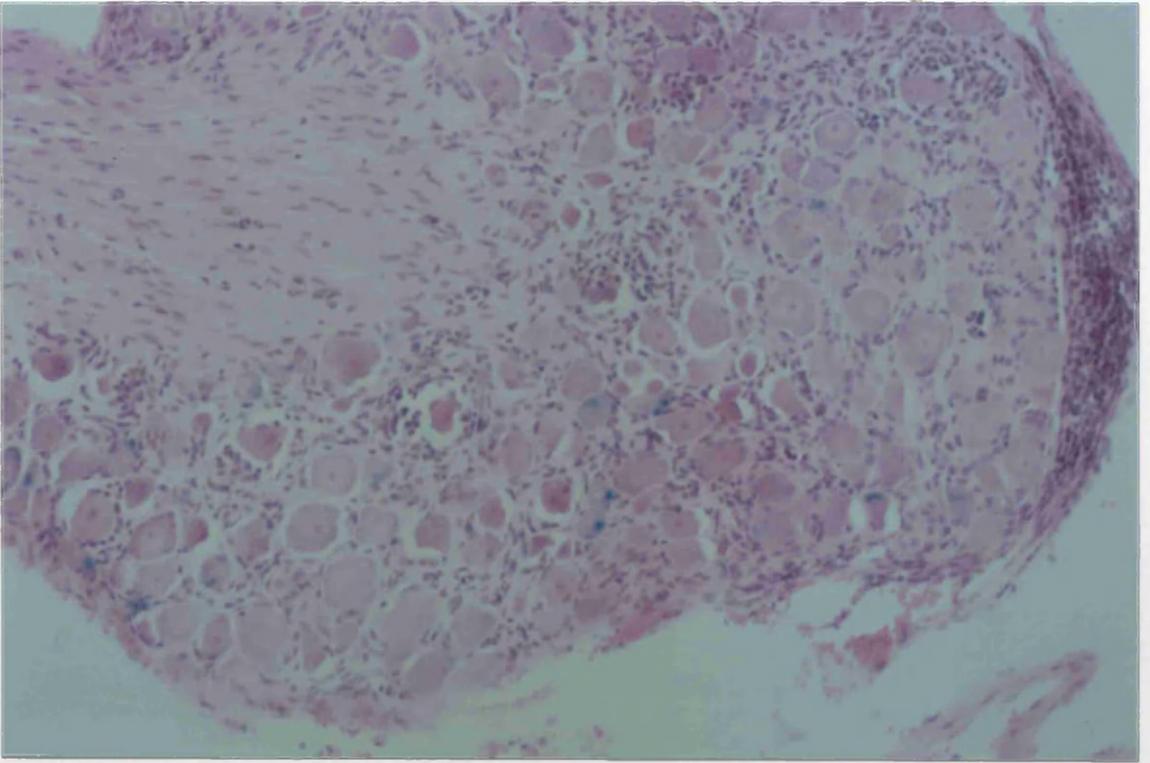
c



50µm

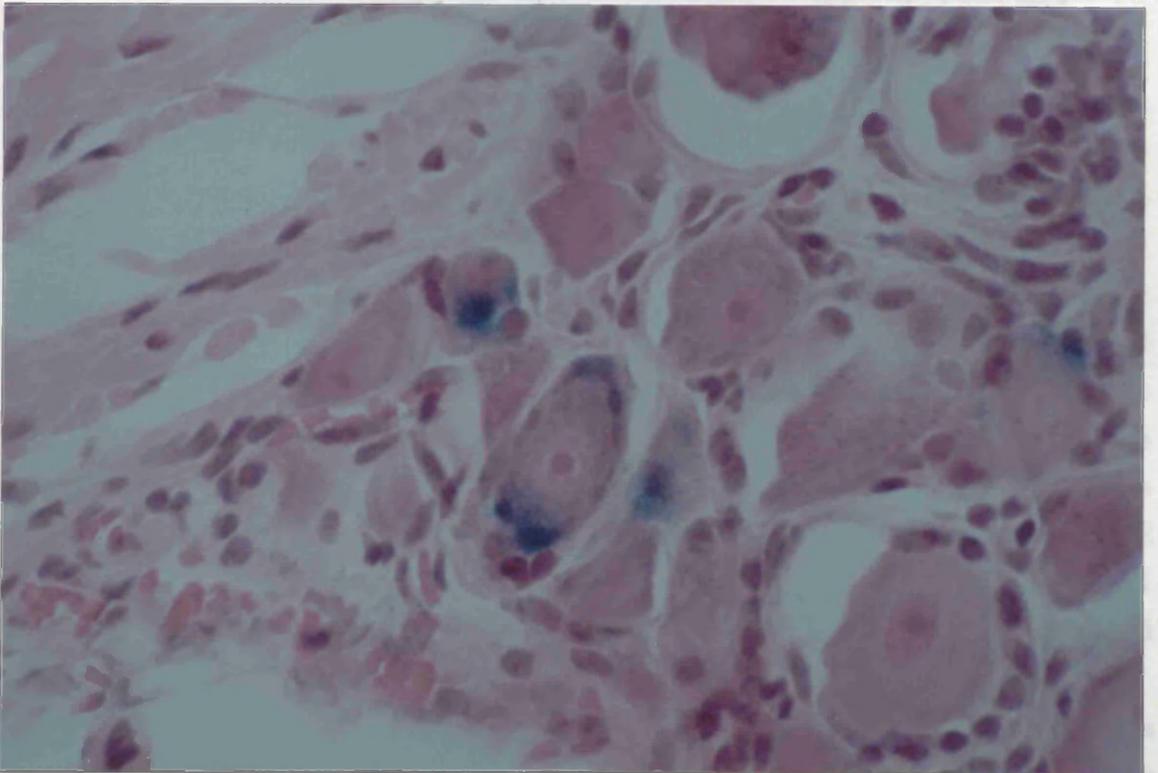
Figure 49. β -galactosidase expression in sections of dorsal root ganglion neurons from mice latently infected with vFJ12. At 6 months after inoculation, animals were sacrificed and spinal ganglia were removed. The ganglia were histochemically stained for β -galactosidase activity, paraffin embedded, and sectioned at 6 μ m. a) Shows a whole section from a ganglion latently infected with vFJ12. Several neurons can be observed with a speckled pattern of cytoplasmic labelling. b) shows a higher magnification of cytoplasmic staining in neurons.

a



150 μm

b



50 μm

were located in the L4 and L5 ganglia (Table 9). Labelling was occasionally observed in the lower thoracic ganglia. The histochemical staining of latently infected neurons was predominantly of a speckled cytoplasmic nature, and of similar intensity to that observed at day 8. Labelling was never observed in axons or non-neuronal cells.

This result shows, perhaps surprisingly, that the *lacZ* gene is expressed in neurons of sensory ganglia latently infected with vFJ12. The implications of this result are discussed in the following section.

5E. DISCUSSION

5E.1. Characterisation of Gene Expression from vFJ12

The aim of this study was to test the animal latency system and histochemical gene expression assay by examining HSV-2 IE-4/5 promoter activity in the context of the HSV-1 genome during acute and latent infection of murine dorsal root ganglion neurons. As described in Section 5D.3, histochemically stained neurons were observed at 4, 6 and 8 days post infection with vFJ12. The most intensely stained neurons were observed at 4 and 6 days which is coincidental with the peak of acute infection (see Section 1D.3.1). This result indicates that the HSV-2 IE-4/5 promoter is capable of exhibiting high expression during acute infection of dorsal root ganglion neurons, and is consistent with the notion of IE promoters working at maximum efficiency during lytic infection. Those neurons observed at 8 days post infection were very much less intensely stained, probably as a result a reduction in IE promoter activity near the end of the acute stage of viral infection. They also appeared speckled as was observed during latency.

The most intriguing result of this study was the observation that vFJ12 is capable of exhibiting long term

expression (up to 6 months) in latently infected sensory ganglia. This seems contrary to the accepted opinion that, with the exception of the LAT gene, the HSV-1 genome is transcriptionally silent (Stevens *et al.*, 1987; Deatly *et al.*, 1988; Gordon *et al.*, 1988). It is possible that the β -galactosidase activity observed in latently infected ganglia is residual from the initial infection. This seems unlikely, however, since on the basis of the difference in the labelling of neurons between days 4 and 6, and day 8 of acute infection, β -galactosidase does not appear to be a particularly stable enzyme. Even if it were stable, it is difficult to conceive of it being present in neurons for up to 6 months. Another possibility is that the virus was in the process of reactivating at the time of dissection, although this again is unlikely due to the fact that histochemical labelling was observed in sensory ganglia from all mice latently infected with vFJ12, and it is highly improbable that all of these mice were undergoing reactivation. β -galactosidase activity has been used as a criterion of promoter activity in latently infected ganglia in a number of other studies (Dobson *et al.*, 1989, 1990; Ho & Mocarski, 1989), and thus supports the view that the β -galactosidase activity observed in sensory ganglia was due to genuine latent expression of *lacZ* from vFJ12. Studies of the genome structure have, however, revealed certain ambiguities in the region surrounding the insertion of IE-4/5 promoter *lacZ* fusion in U_s . As explained in section 5D.4, while initial sequencing studies appear to confirm that the IE-4/5 promoter is still upstream of the *lacZ* gene, restriction profile analysis of the viral DNA has revealed deletions in the U_s region which take out *Bam*HI *x*, and one copy of *Bam*HI *y*. It is possible that this has occurred by homologous recombination between the introduced HSV-2 IE-4/5 promoter and the endogenous HSV-1 IE-4/5 promoters which flank U_s in TR_s and IR_s respectively. Comparison of the HSV-1 and HSV-2 IE-4/5

promoter sequences does indeed reveal some homology (see Figure 50), thus making this prospect a definite possibility. More likely is that the deletion in vFJ12 could have moved the IE-4/5 promoter/*lacZ* fusion into the LAT transcription unit, thereby effectively placing the *lacZ* gene under control of the LAT promoter, with ensuing β -galactosidase activity being observed during latency. As both *Bam*HI *b* and *e* are unaffected by the deletion in vFJ12, it seems most likely that the *lacZ* gene has moved into *Bam*HI *k*. This possibility can only be confirmed by further characterisation of the vFJ12 genome, most likely by sequencing from the HSV-2 IE-4/5 promoter into the flanking endogenous HSV-1 sequence. If this were the case though, then a precise determination of where *lacZ* is inserted into the LAT gene in vFJ12 could provide valuable information concerning the size and nature of the LAT transcript.

Although the majority of other studies have, with the exception of LAT, failed to detect gene expression from the latent HSV-1 genome, it is interesting to note that Deatly *et al.* (1987), when using *Bam*HI *n* and *Bam*HI *x* probes (specific for the IE-3 and 4, and IE-5 genes respectively) could detect a limited number of strongly positive cells in latently infected mice. Although they scored these sections overall as negative, they stated that transcripts from these genes may be present at very low levels and/or expressed in a small fraction of latently infected cells. This would fit in with the comparatively low (compared with that during acute infection) β -galactosidase activity observed in sensory ganglia latently infected with vFJ12.

All other studies have, as yet, failed to detect expression from any of the other IE genes during latency. Deatly *et al.* (1988) and Gordon *et al.* (1988) failed to detect any IE-1, 2 or 3 transcripts using *in situ* hybridisation, and Lynas *et al.* (1989) failed to detect IE-1 gene expression using the polymerase chain reaction. In another study, Ho & Mocarski, (1989), when using a

HSV-1 recombinant containing an IE-3 promoter/*lacZ* fusion, could detect strong β -galactosidase activity in murine sensory ganglia during acute infection, but did not observe any long term expression during latency.

5E.2. Applications of HSV-1 Based Vectors

The demonstration that recombinant herpes simplex viruses are able to express foreign genes under the control of viral and heterologous promoters during latent infection of PNS and CNS neurons (Dobson *et al*, 1990), raises the possibility for the use of such vectors in neurobiological investigations. For example, as proposed earlier, a vector expressing the whole neurofilament gene under control of its own or the LAT promoter could be used to study the interaction of NF proteins with the neuron cytoskeleton. Also, as Geller & Freese (1990), suggested, HSV vectors could be used to alter the physiology of neurons by transduction of genes encoding second messengers or neurotransmitters.

Perhaps the most exciting potential application of herpes vectors is in human gene therapy in the brain. Several pioneering gene therapy studies have been carried out involving retrovirus-mediated gene transfer of genetic material into cells derived from a variety of somatic host tissues, including cells from the haemopoietic system, fibroblasts, hepatocytes, endothelial cells and myoblasts (reviewed in Verma, 1990). Retroviral-mediated gene transfer in the nervous system is not feasible, however, since retroviruses cannot integrate their DNA into non-mitotic cells such as neurons. HSV on the other hand, is capable of long term retention of its genome in neurons, and of expressing foreign genes while latent. Potential problems that may have to be overcome are, for example, establishing that any promoters used to drive gene expression are active in the CNS, and also how efficiently the virus establishes latency when inoculated directly into

the brain. Additionally, it is possible that straightforward neuronal expression *per se* of any transduced gene may not be sufficient, and control of expression may have to be considered.

Two neurological disorders which can be considered as candidates for herpesvirus based gene transfer therapy are Lesh-Nyhan syndrome and Parkinson's disease. The former is a devastating disease caused by a deficiency of the purine salvage enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) which catalyses the conversion of hypoxanthine and guanine to the mononucleotides IMP and GMP respectively. This leads to the build up of uric acid culminating in severe neurological disease. Pallela *et al.* (1988), have reported HSV-mediated HGPRT gene transfer into a HGPRT-deficient neuroma cell line. Their virus had the HGPRT gene inserted into the TK locus of the HSV-1 genome, and under control of the TK promoter. This particular construct would therefore not be useful for *in vivo* studies since the TK promoter is not functional during latency. The HGPRT gene would have to be expressed from a neuronally active promoter, eg, the LAT or perhaps from its own promoter.

Parkinson's disease is caused, at least in part, by failure of neurons of the substantia nigra to synthesise the neurotransmitter dopamine. Currently, the most effective treatment of the symptoms of Parkinson's disease is L-dopa, the precursor of dopamine, the systemic administration of which results in undesirable side effects. A more effective therapy would perhaps be the use of a HSV vector to transduce the gene encoding tyrosine hydroxylase (the enzyme that converts tyrosine to L-dopa) into neurons of the substantia nigra, thereby restoring the ability to produce dopamine directly to appropriate target areas within the brain. Another therapy being considered involves intracerebral grafting of primary fibroblasts genetically engineered to produce L-dopa by retrovirus-

mediated gene transfer. Fisher *et al.* (1991) demonstrated that fibroblasts transduced with the tyrosine hydroxylase cDNA or β -galactosidase gene continued to express the transgene for at least 10 weeks. Tyrosine hydroxylase was also synthesised by fibroblasts implanted into a host (rat) brain as assessed by a behavioural measurement of an animal model of the disease. Graft efficacy however, diminished between 4 and 8 weeks post grafting, a possible reason for which was the shut-off of tyrosine hydroxylase gene expression through loss of MMTV LTR promoter function. Loss of LTR function has been observed in other retroviral vectors (Xu *et al.*, 1989), as well as in a HSV-1 based vectors in motor neurons (Dobson *et al.*, 1990). Transient expression has also been observed with a HSV-1 based vector containing a CMV major IE promoter/*lacZ* fusion in cultured neurons (Johnson *et al.*, 1992). It thus seems that further improvements are required in the design of HSV vectors to allow long-term gene transfer to neurons, both *in vitro* and *in vivo*.

The notion of HSV-1 mediated gene transfer in the nervous system must, however, be treated with reserve, since it is quite clear that a greater understanding of the mechanisms underlying the pathogenesis of latent HSV infection is required before any potential gene therapy vector can be put to serious use. Such a vector will probably have to be delivered directly to target neurons in the CNS, and will therefore have to be rendered incapable of entering the lytic cycle, thus establishing a permanent unreactivable latent infection. Furthermore, the vector could not be cytotoxic, as certain viruses with mutations preventing entry into the lytic cycle, (eg *tsk* at *npt*) are still capable of being. Finally there is the possibility that HSV can cause transformation and oncogenesis, though this has never been shown for cells in the nervous system. Nevertheless, despite these important issues, all of which could be overcome in due course, it is certain that HSV

vectors have tremendous potential, and it will be of great interest to see what further developments can bring to this field.

4F.4. Future Prospects

The demonstration that *lacZ* can be used as an effective reporter gene to evaluate promoter function during latency is certain to aid the understanding of the control of gene expression from the latent HSV genome, especially with respect to analysing the LAT promoter. Indeed, plasmids have been constructed which have portions of the LAT promoter (the LAT6 and LAT60 fragments) driving *lacZ* activity and these await insertion into the virus and subsequent characterisation. This study should allow a definitive characterisation of the minimal sequence required for LAT promoter function during latency, with subsequent mutagenesis being carried out to define and determine the importance of critical sequence motifs.

In addition to determining why certain promoters are functional during latency, the vectors can also be employed to determine why the HSV lytic cycle promoters are non-functional. As well as deletion and site-directed mutational analysis, it would be interesting to swap sequences between the LAT and lytic cycle promoters. An additional virus which should aid this analysis, vFJ7Gal with the HSV-2 RR₂ driving *lacZ* gene expression has been constructed and awaits characterisation.

In addition to experiments characterising promoter functionality during latency, recombinants could be constructed where the reporter gene is replaced by other genes of greater interest. As well as the NF gene suggested earlier (section 4F.3), the coding sequences of other HSV genes, in particular the IE genes, could be used, and these viruses characterised for their ability to establish, maintain and reactivate from latency. Continuous expression of IE genes from the viral genome in neurons, however,

carries inherent dangers, in as much that they may prevent HSV from entering the latent state and going on to cause neuronal destruction with potentially lethal consequences. These vectors may also allow us to address questions such as whether or not a latently infected neuron can be further infected following a secondary application of HSV in mice, or if all latent HSV-1 genomes containing intact LAT transcription units actually express LAT. To answer the former question, one could establish a latent infection with a LAT⁻, β -galactosidase expressing virus, followed by superinfection with a LAT⁺ virus. Alternative sections of latently infected ganglia could then be processed for histochemical staining for β -galactosidase activity, and *in situ* analysis for LAT respectively. Doubly-infected neurons could subsequently be identified by comparison of alternate sections. With respect to the latter question, one could establish a latent infection with a virus that expresses β -galactosidase (though not under control of the LAT promoter) and again process alternative sections of latently infected ganglia for histochemical and *in situ* analysis. The identification of neurons which express *lacZ*, but not LAT, would be an indication that not all latently infected genomes express LAT. It may be, however, that genomes not able to express LAT may also not be able to express *lacZ*.

Finally, a comprehensive study of the latency and pathogenesis characteristics of 1802/1804 is required. Of possible interest here is the apparent correlation between non-pathogenicity and reduction in expression of the 21K protein encoded by *Us11* when sequences are inserted into the *XbaI* site. It will be interesting to see if further studies can determine a direct role for this gene, of as yet unknown function, in virus pathogenesis.

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-501 GGGGGTTGCGGTTTCTGTTTCTTTAAACCCGCTCTGGGGTGTTTTTCGTTCCGTCGCCGGAA

SmaI
-441 TGTTTCGTTTCGTCTGTCCCTCACGGGGCGAAGGCCGCGTACGG**CCCGGG**ACGAGGGGCC
<----- Conserved

NaeI
-381 CCCGACCGCGGCGGTCCGGGCCCCGTCCGGACCCGCTC**GCCGGC**ACGCGACGCGAAAAAG
locus 7 -----> <-----

-321 GCCCCCGGAGGCTTTTCCGGGTTCCCGGCCCGGGCCTGAGATGAACACTCGGGGTAC
---- Conserved locus 8 ----->

NaeI
-261 CGCCAACG**GCCGGC**CCCCGTGGCGGCCCGGCCCGGGCCCCGGCGGACCCAAGGGGCCCC
<----- Conserved locus 9 -----

SphI
-201 GGCCCGGGCCCCACAACGGCCCGGC**GCA**TGCCTGTGGTTTTTTTTTCTCGGTGTTCT
----->

-141 GCCGGGCTCCATCGCCTTTCCTGTTCTCGCTTCTCCCCCCCCCTTCTTACCCCCAGTA

-81 CCCTCCTCCCTCCCTTCCCTCCCCGTTATCCCACTCGTCGAGGGCGCCCCGGTGTGCTTC

|-- 5' end of 2 kb LAT --->
-21 AACAAAGACGCCGCGTTTCCAGGTAGGTTAGACACCTGCTTCTCCCAATAGAGGGGGG

39 GACCCAAACGACAGGGGGCGCCCCAGAGGCTAAGGTCGGCCACGCCACTCGCGGGTGGGC

HinfI
99 TCGTGTTACAGCACACCAGCCCGTTCTTTTCCCCCCTCCACCCCTTAGTCAG**ACTC**TGT

NaeI
159 TACTTACCCGTCGACCACCAACTGCCCCCTTATCTAAGG**GCCGGC**TGGAAGACCGCCAG

219 GGGGTCGGCCGGTGTGCTG

