MOLECULAR ANALYSIS OF HERPES SIMPLEX VIRUS TYPE 1 LATENCY IN EXPERIMENTALLY INFECTED MICE

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ABSTRACT

The molecular characteristics of herpes simplex virus (HSV) latency were studied using an experimental model that makes novel use of the segmental sensory innervation of mouse flanks. Sensory dorsal root ganglia from consecutive spinal segments of C57BL10 mice, latently infected with a virulent strain of HSV-1 (SC16), were compared with respect to (i) HSV DNA levels (by Southern blot hybridization), (ii) latency associated transcripts (LATs, by northern blot hybridization), and (iii) number of LAT^+ neurons (by *in situ* hybridization). Two patterns of HSV persistence could be distinguished, dependent on the anatomical location of ganglia with respect to the site of cutaneous inoculation. The bulk of the viral DNA recovered from latently infected spinal ganglia did not correspond anatomically with the greatest amount of LATs or the highest prevalence of LAT^+ neurons. Viral DNA was most abundant in spinal ganglia directly innervating the inoculation site and the amount recovered suggested that LAT^+ neurons each contain hundreds (approx. 200) copies of HSV DNA. In stark contrast, although LATs and LAT^+ neurons were most abundant in neighbouring ganglia, viral DNA was scarce (approx. 20 copies/LAT^+ cell). These data imply that most of the SC16 DNA detected in ganglia innervating the site of inoculation is transcriptionally repressed. When amplification of HSV DNA during the establishment phase in neurons was prevented by infecting mice with a viral thymidine kinase deletion mutant, the high copy pattern was eliminated and each LAT^+ neuron contained, on average, 22 viral genomes. It was concluded that 'input' (ie. unamplified) and progeny (ie. amplified) DNA sequences persist in the nervous systems of mice infected with SC16. Structurally, latent TKDM21 DNA lacked free genomic termini, consistent with persistence of 'input' DNA in an integrated or circular episomal configuration.

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

BARRY SLOBEDMAN

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ABBREVIATIONS

ATP	adenosine-5'-triphosphate
bp	base pair
Ci	curie
cpm	counts per minute
CTP	cytidine-5'-triphosphate
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DDW	double distilled water
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dpm	decays per minute
DTT	dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
g	gravity
GTP	guanosine-5'-triphosphate
hr	hour
kb	kilobase
kDa	kilodalton
M	molar
mg	milligram
min	minute

ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
nm	nanometre
pfu	plaque forming unit
rATP	2'-ribose-adenosine-5'-triphosphate
rCTP	2'-ribose-cytidine-5'-triphosphate
rGTP	2'-ribose-guanosine-5'-triphosphate
RNA	ribonucleic acid
RNAse	ribonuclease
rUTP	2'-ribose-uridine-5'-triphosphate
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
u	unit
UTP	uridine-5'-triphosphate
w/v	weight per volume
% G+C	percentage guanosine and cytidine
μCi	microcurie
μg	microgram
μm	micrometre
μM	micromolar
^{0}C	degrees celsius

PUBLICATIONS/PRESENTATIONS ARISING

(copies of original papers located in appendix)

Slobedman, B., Efstathiou, S. & Simmons, A. (1994). Quantitative analysis of herpes simplex virus DNA and transcriptional activity in ganglia of mice latently infected with wild type and thymidine kinase deficient viral strains. *Journal of General Virology (in press).*

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1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 The Family herpesviridae

There are approximately 100 known herpesviruses which infect a wide range of vertebrates including humans and other mammals, birds, reptiles, amphibians and fish (Roizman, 1982). They are ubiquitous and highly successful viruses that vary enormously in their pathology and biology (Roizman, 1990).

1.1.1 Properties of herpesviruses

All herpesviruses share a common virion structure comprised of (a) a core containing linear, double stranded DNA (Furlong *et al.*, 1972) (b) an icosahedral capsid made up of 162 capsomers and approximately 100 nm in diameter (Wildy *et al.*, 1960, Horne and Wildy, 1961) (c) the tegument, which is an electron-dense globular substance surrounding the capsid and is often distributed asymmetrically (Morgan *et al.*, 1968) and (d) a lipid envelope with a trilaminar appearance composed of altered cellular membranes and containing many surface glycoprotein projections (Morgan *et al.*, 1959; Epstein, 1962; Stannard *et al.,* 1987).

Herpesviruses are characterized by their ability to encode a large array of enzymes involved in nucleic acid metabolism, synthesis of viral DNA, capsid assembly in the nucleus, destruction of infected cells *in vitro* and persistence for an extended period of time in a latent state in their natural hosts following primary infection (Wildy *et al.*, 1982; Roizman and Sears, 1987).

1.1.2 Classification

Based on host range, duration of reproductive cycle, cytopathology and characteristics of latent infection, herpesviruses have been classified into three sub-families; alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. In addition, some herpesviruses have been further classified into genera based on genome structure, serological relationship and DNA sequence homology (Roizman *et al.,* 1981).

1.1.2.1 Alphaherpesvirinae

Alphaherpesviruses are epidermo-neurotropic viruses that have a broad host range, short growth cycle (less than 18 hours *in vitro*) and the ability to spread rapidly (Darlington and Granoff, 1973). The primary structure of genomes in this sub-group are diverse, having a large variation in G+C content (Honess, 1984; Roizman and Batterson, 1984). They cause productive cytocidal infections in a wide range of cell and tissue types. Typically these viruses cause a mild primary infection of epithelial cells of the skin, eyes, oral cavity and respiratory and genital tracts, resulting in vesicular lesions. Many are

known to establish a latent infection in sensory ganglia (Wildy *et al.*, 1982), although some evidence suggests that virus may also persist in peripheral tissues (Scriba, 1977; Hill *et al.*, 1980; Openshaw, 1983; Cook *et al.*, 1987; Clements and Subak-Sharpe, 1988; Claoue *et al.*, 1990). Alphaherpesviruses incorporate the genera *Simplexvirus* (herpes simplex virus type 1, HSV-1; herpes simplex virus type 2, HSV-2; circopithecine herpesvirus 1, bovine mamillitis virus) and *Varicellovirus* (varicella-zoster virus, VZV; pseudorabies virus, PSV; equine herpesvirus type 1, EHV-1) of which the natural host for HSV-1, HSV-2 and VZV are humans (Clements, 1993).

1.1.2.2 Betaherpesvirinae

Betaherpesviruses are characterized by a narrow host range that is generally species specific *in vitro* and *in vivo* (Wright, 1973). The viral reproductive cycle in culture is long and virus spread is slow. Infected cells often become enlarged (cytomegalia), cytoplasmic and nuclear inclusions are frequently observed and carrier cultures can be readily established (Roizman *et al.*, 1981). Betaherpesviruses have been isolated from a wide range of vertebrates including mice, monkeys and humans; they can remain latent in secretory gland epithelial cells, lymphoreticular cells, kidneys and other tissues (Clements, 1993). In contrast to alphaherpesviruses there is no evidence for persistence of betaherpesviruses in the nervous system. Betaherpesviruses include the genera *Muromegalovirus* and *Cytomegalovirus.* They produce ubiquitous, persistent infections in asymptomatic adults but can produce

disseminated infections involving a wide range of tissues in neonates and immuno-compromised individuals. Three viruses from this subgroup; human cytomegalovirus (HCMV), human herpesvirus type 6 (HHV-6) and human herpesvirus type 7 (HHV-7 or RK virus) have humans as their natural host (Salahuddin *et al.*, 1986; Frenkel *et al.*, 1990; Ho, 1991).

1.1.2.3 Gammaherpesvirinae

Gammaherpesviruses are associated with lymphoproliferative diseases and have a host range that is restricted to the phylogenetic family or order to which the natural host belongs. All members of this sub-family can infect lymphoblastoid cells *in vitro* and the replicative cycle is often halted without production of progeny virus (Craighead *et al.*, 1972; Roizman *et al.*, 1981). Some viruses can cause lytic infections in certain epithelial and fibroblastic cells, and latent infection can be established in lymphoid tissue. Gammaherpesviruses are specific for either B or T lymphocytes and on this basis they are divided into two subgroups; γ_1 and γ_2 (Deinhardt *et al.*, 1974; Frank *et al.*, 1976). Epstein-Barr virus (EBV) and related viruses of Old World monkeys and apes, which make up the γ_1 subgroup, are typically associated with B-lymphocytes and immortalize these cells *in vitro* to produce cultures in which multiple copies of the virus genome are maintained as circular episomes with restricted virus gene expression. The γ_2 herpesviruses include herpesvirus saimiri and other viruses of New World monkeys and lower vertebrates, which persist as inapparent infections of T-lymphocytes in the majority of adult members of

their natural host species. Some members of the γ_2 subgroup can cause lymphoproliferative diseases as well as immortalize T-lymphocytes *in vitro* and in such cultures multiple-copies of the virus genome can persist as circular episomes with limited gene expression (Honess, 1984; Kieff and Leibowitz, 1990). EBV is the only gammaherpesvirus that naturally infects humans.

1.1.2.4 Further considerations

Although herpesviruses have been classified into sub-families based primarily on biological criteria, a genetic basis for classification has gathered momentum as DNA sequence data on a wider range of herpesviruses has become available. The objective criteria available are (i) the conservation and arrangement of genes and gene clusters, (ii) the arrangement of terminal sequences associated with viral genome packaging, and (iii) the distribution of nucleotides that are subject to methylation (Roizman, 1990). There is a strong correlation between biological and genetic criteria with respect to grouping the herpesviruses into three sub-families. However, there are two notable exceptions; (i) Mareks disease herpesvirus is classified as a member of the gammaherpesvirinae based upon biological properties yet falls into the alphaherpesvirinae by some objective criteria (ie. conservation and collinearity of gene clusters) (Buckmaster *et al.*, 1988) and (ii) HHV-6 which by biological criteria should be classified into the gammaherpesvirinae yet by objective criteria is classified as a member of the betaherpesvirinae (Roizman, 1990).

As the genetic information on different herpesviruses increases, so too will any inadequacies in the current system of classification. It is clear, however, that the are three sub-families differ in basic biological properties and that delineation and evolutionary relatedness of genes responsible for these biological properties, when understood, may be the most suitable basis for herpesvirus classification.

1.2 Herpes simplex virus

Herpes simplex virus (HSV) is an important human pathogen that infects many people throughout the world. Like other herpesviruses, HSV can enter a latent phase following productive infection, and from time to time can reactivate from latency to produce new infectious virus. Although HSV has been studied extensively, the molecular basis of infection, particularly the establishment, maintenance, and reactivation from latency, remain poorly understood.

Based upon serological criteria, there are two distinct types of HSV, type 1 (HSV-1) and type 2 (HSV-2). HSV-1 is associated primarily with infections of the head and neck, whereas HSV-2 predominantly infects genital and anal regions (Dowdle *et al.*, 1967).

1.2.1 History

The word 'herpes' is derived from the Greek word *herpo*, meaning 'to creep', and has been used as a medical term for at least 2500 years (Beswick, 1962). For many centuries the term was used to describe a range of skin diseases including eczema and skin cancer and it was only in the 17th century that specific reference was made to herpes febrilis (cold sores). Several reports relating to the classification and systematization of various herpesviruses appeared during the 18th century, but the modern notion of herpes probably began with the definitions of Willan and Bateman in 1814 who differentiated labial and genital herpes from herpes zoster (Bateman, 1814). In 1873 HSV was shown to be able to infect humans (Vidal, 1873) and in 1920 Gruter reported successful transmission of HSV from humans to rabbits. HSV was shown to be a filterable agent in 1921 by Luger and Lauda. Significant advances in the understanding of HSV infection were made during the 1930's when the following observations were reported: (i) Andrews and Carmichael in 1930 found that recurrent herpes occurred only in persons that were positive for herpesvirus neutralizing antibody, (ii) in 1938 Doerr suggested that herpes simplex might be endogenously generated in the host by unknown means, and (iii) Burnet and Williams in 1939 distinguished primary from recurrent disease and hypothesized that primary infection led to life-long persistence of virus in ganglia. It wasn't until 1961 that Shneweis and Brandis discovered two distinct antigenic types of HSV and in 1967 the anatomical site of infection and antigenic type were shown to generally correlate (Dowdle *et al.*, 1967).

1.2.2 Epidemiology

HSV infection is extremely common and widespread and is not limited by geographical, racial or seasonal boundaries. The prevalence of infection is the same in males and females (Nahmias *et al.*, 1989). Humans are thought to be the sole reservoir of infection ie. no animal vectors have been identified. Transmission is by close personal contact between susceptible individuals and primary infection is usually associated with mucosal or skin surfaces (Roizman, 1990). Infection is rarely fatal and consequently the pool of latently infected humans includes a large proportion of the world's population. Seroepidemiological studies in the 1940's and 1950's demonstrated that up to 90% of the population has antibodies to HSV by the fourth decade of life (Nahmias and Roizman, 1973). Seropositivity is indicative of exposure to the virus because primary HSV infection results in the production of long-lasting neutralizing antibodies.

Although HSV-1 and HSV-2 overlap considerably in terms of epidemiology, they tend to infect different regions of the body. Primary infection with HSV-1 is predominantly oral and/or perioral and kissing is the most common mode of transmission. However, HSV-1 can infect the eye, genitals and other organs. The prevalence of HSV-1 antibodies displays two peaks with respect to age; one in the first few years of life, so that more than 50% of most populations are seropositive by the age of 1 year, and a second peak coinciding with puberty, when seropositivity rises to between 60 and 85% (Nahmias *et al.*,

1970a). The highest prevalence of HSV antibodies is found in lower socio-economic groups. Primary infection with HSV-2 occurs mostly in the genital region, is usually acquired from puberty onwards, and is transmitted primarily by sexual contact (Kaufman *et al.*, 1973). The prevalence of HSV-2 antibodies varies considerably between populations, ranging from 10% in Americans to 77% in Ugandans (Rawls *et al.*, 1972) and epidemiological factors relating to sexual activity appear to have a strong influence on the extent of HSV-2 infection.

The ability of HSV to latently infect the majority of the population and to reactivate frequently provides the virus with a large reservoir for transmission to susceptible hosts. Significantly, both HSV-1 and HSV-2 can be excreted in the absence of clinical symptoms during primary or recurrent infection (Buddingh *et al.*, 1953; Haynes *et al.*, 1968; Rattray *et al.*, 1978; Ekwo *et al.*, 1979; Hatherly *et al.*, 1980). Therefore, there also exists a silent reservoir for the transmission of infection.

1.2.3 Clinical manifestations

HSV is primarily associated with orofacial and genital disease but the virus also causes ocular disease, encephalitis and neonatal herpes. Recurrences are confined to the dermatome of the primary infection (Spruance *et al.*, 1977) and the symptoms of recrudescence tend to be less severe than those of primary infection.

Primary orofacial infections are often asymptomatic but clinical symptoms can include fever, sore throat, ulcerative and vesicular lesions, oedema, localized lymphadenopathy, gingivostomatitis, anorexia and malaise (Kohl, 1987). Clinical illness of 2-3 weeks duration follows an incubation period of 2-12 days (Corey *et al.*, 1983). Recurrent infection is often preceded by prodromal symptoms such as pain, burning, tingling or itching which lasts for approximately 6 hr. Within 24-48 hr of the prodrome, vesicles form and the lesion progresses to a pustular, ulcerative or crusting stage over the ensuing 72-96 hr (Spruance *et al.*, 1977; Spruance and Crumpacker, 1982). Infectious virus is usually lost after 2-3 days and the lesion heals in 8-10 days (Bader *et al.*, 1978). The factors that induce recurrence include fever, stress, trauma and exposure to ultraviolet light (Ship *et al.*, 1967). The molecular basis of recurrence is poorly understood.

In addition to local blistering and ulceration of the genital tract, symptoms of primary genital infection may include fever, dysuria, malaise, inguinal lymphadenopathy and aseptic meningitis. However, genital infection is often asymptomatic (Rattray *et al.*, 1978; Adam *et al.*, 1979; Corey, 1988) and less than 25% of HSV-2 seropositive humans are aware that they are infected (Stavraky *et al.*, 1983; Becker *et al.*, 1986). Virus can be excreted from the genital tract at a high concentration for about 3 weeks during primary infection. Recurrent infection lasts for 7-10 days during which virus is shed for the first 2-5 days, at a lower concentration than during the primary infection

(Guinan *et al.*, 1981; Silvestri *et al.*, 1982).

Neonatal HSV infection is usually acquired during child birth from primary genital lesions in the mother. However, acquisition can also occur in utero and postnatally eg. oral lesions of the mother or father (Nahmias *et al.*, 1976). Most neonatal infections are caused by HSV-2 (Nahmias *et al.*, 1967) and the consequences can be grave. Infection tends to disseminate, causing widespread destruction of most organs (Hass, 1935; Hanshaw and Dudgeon, 1978). Milder infections affecting only the skin, eyes and mouth can also occur. The incidence of neonatal herpes is much lower than genital HSV infections in the adult child-bearing population.

HSV diseases of the central nervous system (CNS) are extremely serious and quite often fatal and survivors are frequently left with residual neurological abnormalities. Symptoms include fever, altered conciousness, bizarre behaviour, disordered mentation and localized neurological findings. In addition to encephalitis, HSV can involve most, if not all areas of the nervous system (Olson *et al.*, 1967). Immunocompromised individuals are at highest risk of HSV encephalitis as they are for all HSV infections (Pass *et al.*, 1979; Whitley *et al.*, 1984).

People with skin abrasions or burns are also particularly susceptible to HSV-1 and HSV-2 infection. HSV also causes herpetic whitlow, an infection of the digits (Gill *et al.*, 1988), eczema herpeticum in patients with underlying atopic dermatitis and herpes gladiatorium which is found among wrestlers and is spread through skin abrasions (Selling and Kibrick, 1964; Wheeler and Cabraniss, 1965). HSV keratoconjunctivitis is both a frequent and serious infection usually caused by HSV-1 (beyond newborn age). It often results in corneal scarring and is one of the most common causes of blindness in developed countries (Dawson and Togni, 1976). HSV can also cause proctitis among homosexual men (Quinn *et al.*, 1981, Goodell *et al.*, 1983) and herpetic tracheobronchospasm in elderly immunocompetent individuals (Sherry *et al.*, 1988).

1.2.4 Description of the virion

The HSV virion contains linear, double-stranded DNA packaged within a regular protein shell called the capsid (Schrag *et al.*, 1989). The virion is enveloped and the region between the capsid and the envelope is called the tegument. Until recently, viral DNA was thought to be wound on a fibrillar spool, but it now appears certain that it fills the entire capsid and is packed in a locally ordered manner, with bundles of parallel duplex strands (Booy *et al.*, 1991). The genome is approximately 152000 bp in length with a G+C content of 68% (HSV-1) or 154000 bp and 69% G+C (HSV-2) (Becker *et al.*, 1968; Kieff *et al.*, 1971). HSV-1 has been sequenced and encodes at least 73 unique

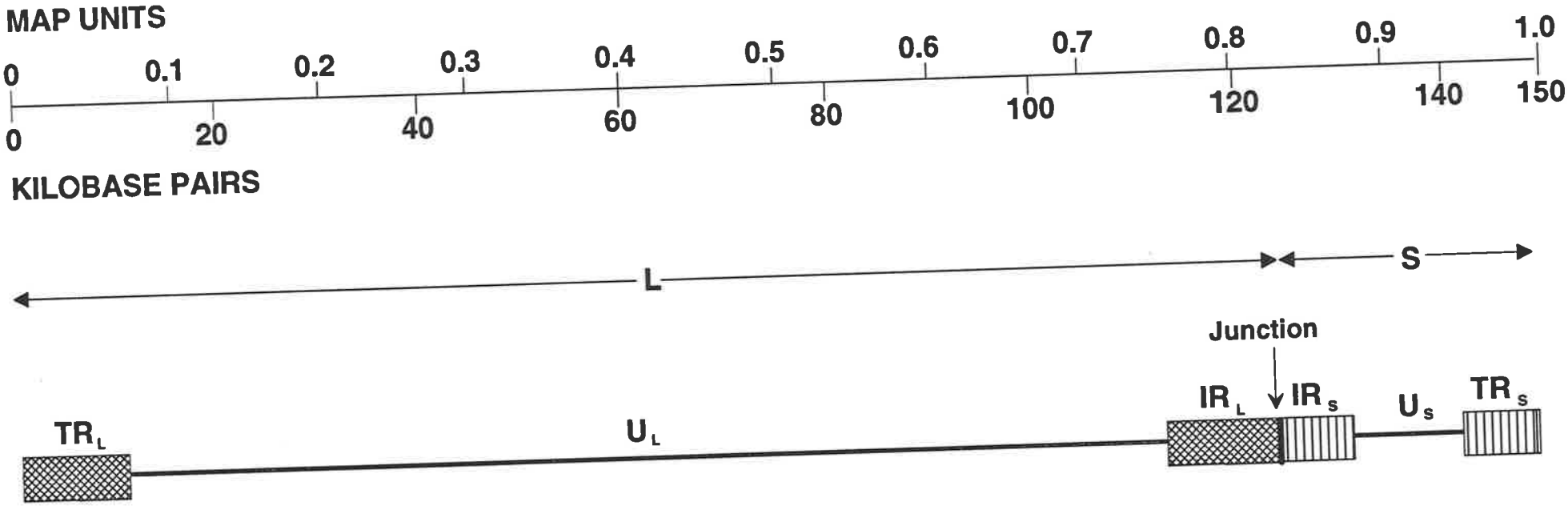
proteins, of which about 28 have an unknown function (rev. McGeoch *et al.*, 1993). The genome consists of covalently linked long (L) and short (S) segments. Each segment consists of a unique region (U_L and U_S) flanked by inverted repeat sequences located internally (IR_L or *b'a'* and IR_S or *a'c'*) and terminally (TR_L or *ab* and TR_S or *ca*) (Sheldrick and Berthelot, 1975; Wadsworth *et al.*, 1975) (Figure 1.1). The *a* component of the repeat regions, which contains sequences which are highly conserved, consists of a variable number of repeat elements (Wagner and Summers, 1978; Roizman, 1979a, b; Davison and Wilkie, 1981). DNA prepared from HSV virions is comprised of equal amounts of 4 isomers, as a result of inversion of the L and S components relative to each other (Hayward *et al.*, 1975; Delius and Clements, 1976; Jacob *et al.*, 1979).

HSV-1 and HSV-2 share approximately 50% of their base pairs and have very similar sequence arrangements, but they differ in many restriction endonuclease cleavage sites (Kieff *et al.*, 1971; Cortini and Wilkie, 1978; Morse *et al.*, 1977; McGeoch 1987).

At least 33 virally encoded polypeptides, but no host proteins, are known to be contained within the HSV-1 virion (Spear and Roizman, 1972; Heine *et al.*, 1974; Roizman and Sears, 1990). No less than 7 of these proteins are thought to be capsid components (Marsden *et al.*, 1987; Heine *et al.*, 1974). A further 10 have been shown to be glycosylated and located on the surface of the virion

Figure 1.1

Map of the HSV-1 genome structure. The genome consists of covalently linked long (L) and short (S) segments. The L segment is comprised of a unique region (U_L) flanked by the long terminal repeat (TR_L) and the long internal repeat (IR_L) regions. The S segment is comprised of a unique region (U_S) flanked by the short terminal repeat (TR_S) and the short internal repeat (IR_S) regions. The junction between the L and S segments is marked.



HSV-1 GENOME STRUCTURE

(Spear, 1993), creating the spikes that can be seen (by electron microscopy) projecting from the envelope by electron microscopy (Stannard *et al.*, 1987). The glycoproteins are termed gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and are thought to be of considerable importance for virus pathogenicity.

The tegument contains the remainder of the virion proteins. Prominent tegument proteins are: (i) A 65 kD protein called *α-trans*-inducing factor (αTIF), which is also known as virion protein (VP) 16; infected cell polypeptide (ICP) 25 and Vmw65. It binds to the viral genome as well as several cellular transcription factors to form a complex called *α-trans*-inducing complex which stimulates the transcription of viral genes (Batterson and Roizman, 1983; Campbell *et al.*, 1984; Pellet *et al.*, 1985; Marsden *et al.*, 1987; Gerster and Roeder, 1988; O'Hare *et al.*, 1988; Preston *et al.*, 1988; Spector *et al.*, 1991) and (ii) the virion host shut-off (VHS) protein, which destabilizes mRNA molecules, thereby shutting down host cell protein synthesis (Nishioka and Silverstein, 1977, 1978; Read and Frenkel, 1983; Kwong *et al.*, 1988).

1.3 Replication of HSV

1.3.1 Attachment and penetration

The first step in the replicative cycle of HSV is attachment of the enveloped virion to a susceptible host cell. Attachment occurs by binding of the virus to cellular receptors which must be widely distributed, because HSV can infect a broad range of hosts (Vahlne *et al.*, 1979; Vahlne *et al.*, 1980). The receptors for HSV-1 and HSV-2 are thought to differ (Vahlne *et al.*, 1980; Addison *et al.* 1984), and cellular heparan sulphate proteoglycans can mediate, at least in part, attachment of virions to the cell surface (Wudunn and Spear, 1989; Shieh *et al.*, 1992; Gruenheid *et al.*, 1993). The principle component for virus attachment to heparan sulphate receptors is gC, yet there must be an alternative, less efficient means of attachment, because gC negative HSV mutants can still infect cells *in vitro* (Herold *et al.*, 1991). gB can also bind to heparan sulphate but it is not required for virus attachment (Cai *et al.*, 1988; Herold *et al.*, 1991)

Attachment induces cytoskeletal changes that trigger microfilament activity, which mediates virus penetration (Rosenthal *et al.*, 1988). Penetration involves fusion between the viral envelope and the cell membrane and subsequent release of the unenveloped capsid into the cytoplasm (Huang and Wagner, 1964; Morgan *et al.*, 1968; Manservigi *et al.*, 1977; Para *et al.*, 1980; Johnson *et al.*, 1984). Although the virus can also enter cells by endocytosis, this

process results in a non-productive infection (Campadelli-Fiume *et al.*, 1988; Wittels and Spear, 1991). Penetration is thought to be a multi-step procedure involving at least three viral glycoproteins. Studies utilizing mutant viruses and neutralizing antibodies have shown that virus penetration requires gB (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Deluca *et al.*, 1982; Highlander *et al.*, 1988; Cai *et al.*, 1988), gD (Fuller and Spear, 1987; Highlander *et al.*, 1987; Johnson and Ligas, 1988; Ligas and Johnson, 1988) and gH (Little *et al.*, 1981; Gompels and Minson, 1986; Desai *et al.*, 1988; Fuller *et al.*, 1989; Forrester *et al.*, 1992). Mutant viruses devoid of these glycoproteins are able to bind to cells at normal efficiency, but fail to penetrate. In addition, gC enhances infectivity because gC negative HSV virions penetrate the cell surface less efficiently than wild-type virus (Herold *et al.*, 1991). Two other glycoproteins, gK and gL, have also been implicated as playing a role in virus penetration (Debroy *et al.*, 1985; Hutchinson *et al.*, 1992).

After penetration, the uncoated viral capsid and tegument proteins are transported through the cytoplasm via the cytoskeleton to nuclear pores, where viral DNA and some tegument proteins are ejected into the nucleus (Tognon *et al.*, 1981; Batterson *et al.*, 1983; Kristensson *et al.*, 1986). Immediately upon entering the nucleus, viral DNA circularizes by covalent joining of the DNA termini (Roizman and Sears, 1987). Circularization is dependent on host factors or viral factors that are components of the virion (Poffenberger and Roizman,

1985). Transcription of viral genes occurs in a coordinately regulated and sequentially ordered manner. Based on timing and the requirements for their expression viral genes can be divided into 3 classes, namely (i) α or immediate-early (IE) genes, (ii) β or early (E) genes and (iii) γ or late (L) genes. Expression of α, β and γ genes results in the production of messenger RNA's (mRNA) that encode α, β, and γ polypeptides respectively (Honess and Roizman, 1973, 1974, 1975; Roizman and Furlong, 1974; Roizman *et al.*, 1974; Fenwick and Roizman, 1977; Jones and Roizman, 1979; Roizman and Batterson, 1984). Transcription of viral DNA is mediated by host cell RNA polymerase II (Costanzo *et al.*, 1977).

1.3.2 Gene expression

α genes: There are 5 α genes, termed 0, 4, 22, 27 and 47 which encode ICP-0, -4, -22, -27 and -47, respectively. The α genes are the first to be expressed and transcription precedes *de novo* synthesis of viral proteins in the infected cell (Honess and Roizman, 1974). All α genes have a characteristic *cis*-acting element found within 400 bp upstream of the cap site (Mackem and Roizman, 1982). This element contains the motif TAATGARAT (where R is a purine residue) which is an essential component of α gene activation (Gaffney *et al.*, 1985). Promoter activity of α genes is induced by the virion tegument protein α-TIF (Roizman and Batterson, 1984; Post *et al.*, 1981; Campbell *et al.*, 1984). Mutations in α-TIF result in attenuated growth *in vitro* and these viruses are avirulent in some animal models (Ace *et al.*, 1989). Although α-TIF is

essential for viral replication (Weinheimer *et al.*, 1992), it does not bind directly to the α gene enhancer element in a stable manner (McKnight *et al.*, 1987). Rather, α-TIF is recruited onto TAATGARAT in a manner dependent on the binding of a ubiquitous cellular DNA-binding transcription factor (OCT-1) (O'Hare and Goding, 1988; O'Hare *et al.*, 1988), which itself can bind to TAATGARAT (O'Hare and Goding, 1988; Stern *et al.*, 1989). The α-TIF binds to and activates OCT-1, redirecting it to target the viral promoter-regulatory domains of α genes containing the TAATGARAT motif (Triezenberg *et al.*, 1988; ap Rhys *et al.*, 1989; Preston *et al.*, 1988). The interaction of OCT-1 and α-TIF is necessary for the transactivation of α genes. Although α-TIF may be able to directly recognize TAATGARAT, effective and stable binding requires the presentation of OCT-1 as well. In addition to the formation of an OCT-1/α-TIF complex, at least one other cellular component (termed CFF) is required for α gene transactivation (Gerster and Roeder, 1988; Xiao and Capone, 1990). Thus, the initiation of α gene transcription is a 4 component system comprising α-TIF, OCT-1, CFF and TAATGARAT, and there may additional recognition sites that augment induced promoter activity (Triezenberg *et al.*, 1988). Further, octamer-like ATGCTAAT motifs that overlap with the TAATGARAT motifs and act as an independent binding site for OCT-1 have been identified in HSV-1 α genes (ap Rhys *et al.*, 1989).

At least 3 α gene polypeptides are involved in the transactivation and/or repression of β and γ gene expression: (i) ICP-4 is essential for the activation and transcription of HSV β and γ genes (Preston, 1979). It also plays an autoregulatory role in that it can bind to its own regulatory sequences, and repress its own synthesis (Roberts *et al.*, 1988). The polypeptide is present in virions and therefore may be required immediately for the initiation of the infectious cycle (Yao and Courtney, 1989). It has a high affinity for DNA and is translocated to the nucleus soon after synthesis where it localizes within viral replication compartments (Pereira *et al.*, 1977). There may be multiple recognition sites for ICP-4 binding and other viral or cellular factors may mediate transactivation (Hay and Ruychan, 1992). (ii) ICP-0 is a promiscuous transactivator ie. it activates many HSV as well as non-HSV genes. This process is enhanced by the presence of ICP-4. ICP-0 is not essential for lytic infection in cell culture during high multiplicity infections, but significant expression of β and γ genes does require ICP-0 after infections at low multiplicity (Sacks and Schaeffer, 1987). Following synthesis, ICP-0 is translocated to the nucleus where it becomes associated with chromatin (Pereira *et al.*, 1977; Hay and Hay, 1980; Knipe, 1989). (iii) ICP-27 acts as a repressor for α and some β genes but is required for the activation of γ genes. As such, it is essential for viral replication. In addition to its role in transactivation, ICP-27 also plays a role in the stabilization and/or export of improperly processed mRNAs (Sandri-Goldin, 1991).

The remaining two α gene polypeptides, ICP-22 and ICP-47 not as well defined in terms of their function. Neither are essential for viral replication (Post *et al.*, 1981; Mavromara-Nazos *et al.*, 1986), but ICP-22 appears to play a role in defining host range and in enhancing expression of some γ genes (Sears *et al.*, 1985a).

The rate of α polypeptide synthesis is greatest 2-4 hr after infection in cultured cells, but these proteins accumulate until later in infection (Honess and Roizman, 1974). α polypeptides may act as targets for cytotoxic T-lymphocytes (Martin *et al.*, 1988, 1990) or for natural killer cell lysis (Fitzgerald-Bocarsly *et al.*, 1991).

β genes: The β genes code for most of the non-structural proteins and they are involved primarily in nucleic acid metabolism (Wu *et al.*, 1988). Two subsets of β genes, termed β_1 and β_2 have been identified. β_1 genes are expressed very early after infection (Roizman and Sears, 1990), slightly overlapping expression of α genes, yet they require a functional ICP-4 before synthesis can begin. β_2 genes encode the majority of the β polypeptides and expression follows that of β_1 genes. There are 7 β gene products that are essential for HSV-1 DNA replication (Challberg, 1986). Many other β gene products are not essential for DNA replication but mutations in these genes often results in attenuated growth (Weller, 1991). The 7 essential replication proteins are encoded by genes located within the U_L region of the viral genome and are

translocated to the nucleus after synthesis. They are: UL9 which encodes an *ori*-binding protein and exhibits DNA helicase activity, UL30 and UL42 which code for two subunits of viral DNA polymerase that, when complexed, make up the functional DNA polymerase, UL5, UL8 and UL52 whose gene products form a complex exhibiting helicase-primase activity, and UL29 which encodes the major DNA-binding protein (commonly called ICP-8). ICP-8 stimulates viral DNA polymerase and helicase activity as well as the organization of replication complexes and DNA polymerase at the onset of DNA synthesis (de Bruyn Kops and Knipe, 1988; Bush *et al.*, 1991). ICP-8 has also been implicated as playing a significant role in regulating gene expression because it down-regulates β polypeptide synthesis and enhances production of some γ polypeptides (Gao and Knipe, 1991). There are five β gene products involved in nucleotide metabolism, including thymidine kinase (UL23) and ribonucleotide reductase (a dimer comprised of the products of UL39 and UL40). All of these enzymes are dispensable for growth *in vitro* but this is not necessarily true *in vivo*. For example, neurons do not support viral replication in the absence of viral thymidine kinase (Tenser and Dunstan, 1979; Tenser *et al.*, 1979). Peak expression of β genes occurs 5-7 hours after infection *in vitro* and the appearance of β gene products signals the onset of viral DNA synthesis (Honess and Roizman, 1974).

γ genes: The γ genes are the final class of viral genes expressed in the HSV replicative cycle. They code for most of the viral structural polypeptides and are divided into the two sub-classes, γ_1 and γ_2, based upon the timing of expression and their requirement for viral DNA replication. The expression of γ_1 genes is stimulated by, but not absolutely dependent on viral DNA replication, and products include gB, gD, α-TIF and the major capsid protein. The γ_1 34.5 gene, which maps within the inverted repeats flanking the long segment of the viral genome, has been shown to enable the virus to multiply and spread in the CNS of mice even though it is not essential for replication in tissue culture (Chou *et al.*, 1990). This gene product also precludes neuroblastoma cells from triggering total shut off of protein synthesis, possibly to enable protein synthesis necessary for viral synthsis (Chou and Roizman, 1992). γ_2 gene expression is almost totally dependent on DNA synthesis and these genes encode polypeptides such as gC and gE. A feature of γ gene expression is stimulation by viral DNA synthesis acting (Mavromara-Nazos and Roizman, 1987). Regulation of γ gene expression requires only the TATA box with surrounding sequences (Homa *et al.*, 1988) and differential expression of γ_1 and γ_2 genes is thought to be a function of TATA element sequences (Homa *et al.*, 1988; Johnson and Everett, 1986). Transactivation and transrepression of γ genes is mediated by both α and β gene products including ICP-4, ICP-0, ICP-22, ICP-27 and ICP-8 (Costa *et al.*, 1985; Everett, 1986; Michael *et al.*, 1988; Sekulovich *et al.*, 1988; Gao and Knipe, 1991). The products of γ genes are synthesized at increasing rates as infection proceeds,

beginning about 9 hours after infection and continuing for at least 3 hours (Honess and Roizman, 1974). The structural and some of the non-structural proteins are transported back into the nucleus where they are required for capsid assembly and DNA packaging.

1.3.3 DNA synthesis

DNA replication begins about 3hours after infection following the onset of β polypeptide synthesis, and continues for 9-12hours (Roizman & Roane, 1964; Roizman *et al.*, 1965; Roizman and Sears, 1990). HSV encodes many proteins required for viral origin-dependent DNA synthesis. These include 7 essential proteins and an array of enzymes that are β gene products. There are three origins of replication in the HSV genome. Two of these, ori_s1 and ori_s2 map within the reiterated sequences of the S component (Mocarski & Roizman, 1982a; Stow, 1982; Stow & McMonagle, 1983; Deb & Doelberg, 1988) and the other, ori_L, maps to the middle of the L component (Weller *et al.*, 1985).

Initiation of DNA replication probably occurs when the UL9 gene product binds to palindromic sequences contained within the origins of replication (Weller *et al.*, 1985; Olivo *et al.*, 1988). Restriction endonuclease and sedimentation studies have suggested that viral DNA progresses from circular and branched linear forms early after the onset of replication to large bodies of tangled DNA later on in the reproductive cycle. These replicative intermediates are high molecular weight concatemeric molecules in which the termini are

fused together in a head-to-tail arrangement (Ben-Porat & Tokazewski, 1977; Jacob & Roizman, 1977; Jacob *et al.,* 1979; Jongeneel & Bachenheimer, 1981). Based on these observations, it has been proposed that DNA replication occurs via a rolling-circle mechanism following circularization of the parental DNA template (Ben-Porat & Tokazewski, 1977; Becker *et al.*, 1978; Jacob *et al.*, 1979; Roizman, 1979a; Poffenberger & Roizman, 1985). Using pulsed-field gel electrophoresis (PFGE), circular viral DNA templates have been indirectly demonstrated in cycloheximide-treated cells (Garber *et al.*, 1993). DNA synthesis is thought to start at a nick at the origin of replication, whereby the replication fork moves continuously around the circular genome to produce head-to-tail concatemers of the monomer genome. Stow (1992) showed that a plasmid containing an HSV origin of replication (ori_s), when transfected into insect cells expressing the seven HSV-1 genes required for DNA replication, was replicated in the form of concatemers. The newly synthesized concatemeric DNA is subsequently cleaved into unit length genomes (Jacob *et al.,* 1979; Vlazny *et al.,* 1982). Cleavage and packaging of progeny genomes into empty capsids is dependent on the *a* sequence (Vlazny *et al.*, 1982; Stow & McMonagle, 1983; Varmuza and Smiley, 1985; Deiss *et al.,* 1986; Deiss and Frenkel, 1986; Smiley *et al.*, 1992).

Isomerization of the viral genome by the inversion of the L and S components relative to each other is associated with the process of DNA replication, cleavage and packaging (Hayward *et al.,* 1975; Delius & Clements, 1976). The

molecular events responsible for the production of equimolar amounts of the 4 isomeric forms is not fully understood. Alternate cleavage of circular or concatemeric DNA can account for the production of only two isomers and it has been suggested that the remaining two isomers are generated by a process of homologous recombination, mediated by the repeated *a* sequences (Mocarski *et al.,* 1980; Smiley *et al.,* 1981; Mocarski & Roizman, 1982a, b; Bruckner *et al.,* 1992; Dutch *et al.,* 1992). In addition to the role of *a* sequences in isomerization there may be additional *cis*-acting sites for inversion within the *b* sequence of the terminal repeats of the L component (Longnecker and Roizman, 1986)

The rolling circle model of viral DNA replication does not alone adequately explain the rapid amplification of viral DNA. Synthesis of one unit length genome is estimated to take 20-40 min. Yet, genomes increase several hundred fold in the first few hours (Jacob & Roizman, 1977). Consequently, it has been hypothesized that plasmid-like amplification of circular templates may precede the initiation of rolling-circle amplification (Hammerschmidt & Sugden, 1990; Hammerschmidt & Mankertz, 1991). Recently Zhang *et al* (1994) used field inversion gel electrophoresis (FIGE) to show that replicative intermediates are concatemers comprised of a head-to-tail arrangement of HSV monomers, in which the long segment of the viral genome is in the opposite orientation in neighbouring genomic units (termed P/I concatemers). From these structures, it is possible, in principle, to produce all 4 isomeric forms by using alternate sites

of cleavage. P/I concatemers were detected at an early stage of DNA synthesis, when replicating intermediates lacked free genomic termini and appeared to be in a circular configuration. Similarily, Severini *et al* (1994) demonstrated inversion of the HSV-1 long segment, in relation to the rest of the viral genome, in concatemers generated after *in vitro* infection. Conventional rolling circle replication cannot account for these observations and the data supports the existence of a different replication strategy bearing strong similarity to that of the yeast 2μ circle (Futcher, 1986). This model involves intragenomic recombination events during DNA replication and bidirectional initiation of DNA synthesis.

1.3.4 Assembly and egress

Assembly of capsids is thought to take place on a cellular protein network and occurs within the nucleus. Viral proteins first form empty capsids before viral DNA is inserted. Further proteins then become associated to this structure to form the complete capsid. ICP-35, the major internal protein constituent, may play a role in the assembly of the capsid shell and in subsequent DNA packaging. This protein undergoes extensive processing (Newcomb and Brown, 1991) that is mediated by a virus-encoded proteinase (Liu and Roizman, 1991a, b). The appearance of thick concave or convex patches in the nuclear membrane signals the initiation of capsid release from the nucleus. These patches are thought to represent aggregations of viral membrane proteins. Capsids associate with regions of the inner nuclear lamella modified by viral

glycoproteins and bud into the perinuclear space. The capsid becomes enveloped during this process (Darlington and Moss, 1969; Morgan *et al.*, 1968; Nii *et al.*, 1968). Envelopment is dependent on the capsid containing a full-sized DNA molecule (Vlazny *et al.*, 1982). Enveloped virions traverse the cytoplasm via the Golgi apparatus, following a similar pathway to that taken by excreted soluble proteins. Finally, virions are released from the cell by exocytosis (Johnson and Spear, 1982). The virus may encode a function mediated by gD that prevents re-infection of cells (Tognon *et al.*, 1981; Campadelli-Fiume *et al.*, 1988). Cell-to-cell spread caused by fusion of infected cells has also been reported with syncytial strains of HSV (Nii *et al.*, 1968). In cultured cells, productive infection ultimately leads to cell death and the complete replicative cycle is very rapid (approximately 18 hours) (Darlington and Granoff, 1973).

1.4 Pathogenesis of HSV infection

The pathogenesis of HSV can be described in terms of the viruses ability to: (i) replicate at the site of infection, accessing nerve endings during the primary infection, (ii) establish a latent infection in sensory neurons, and (iii) cause recurrent infection by reactivating from latency and producing new infectious virus.

1.4.1 Primary infection

In humans, transmission of HSV infection requires close personal contact. Primary HSV infection occurs following exposure of skin or mucous membranes to the virus and may be asymptomatic or result in clinical disease (Mertz *et al.*, 1992). Virus replicates in the parabasal and intermediate epithelial cells resulting in cell death. Characteristically, infected cells balloon and cellular chromatin becomes condensed before nuclei degenerate and cell fusion occurs, leading to the formation of multi-nucleated giant cells (Darlington and Granoff, 1973). Many infected cells contain eosinophilic intranuclear inclusions (Cowdry, 1934). Vesicular fluid contains large numbers of virus particles released by lysed cells, cell debris, inflammatory cells and multi-nucleated giant cells. A dense inflammatory infiltrate is apparent both in the epidermis and in the corium beneath the collection of serous fluid (Rawls, 1985). Mononuclear cells can be detected in infected tissue as host defences are mounted (Whitley, 1990). The typical lesion is a vesicle that forms under the layer of keratinized squamous epithelial cells. After vesiculation, the exudate becomes pustular owing to the recruitment of inflammatory cells. Finally, the lesion dries and a scab forms (Timbury, 1993). Residual scarring can occur, particularly in those individuals that suffer from frequent recurrent lesions (Whitley, 1990). Primary infection of mucous membranes is less likely to result in prominent vesicle formation since the very thin epithelium facilitates rapid formation of shallow ulcers. Draining lymph nodes often become enlarged with an influx of inflammatory cells as a result of spread of

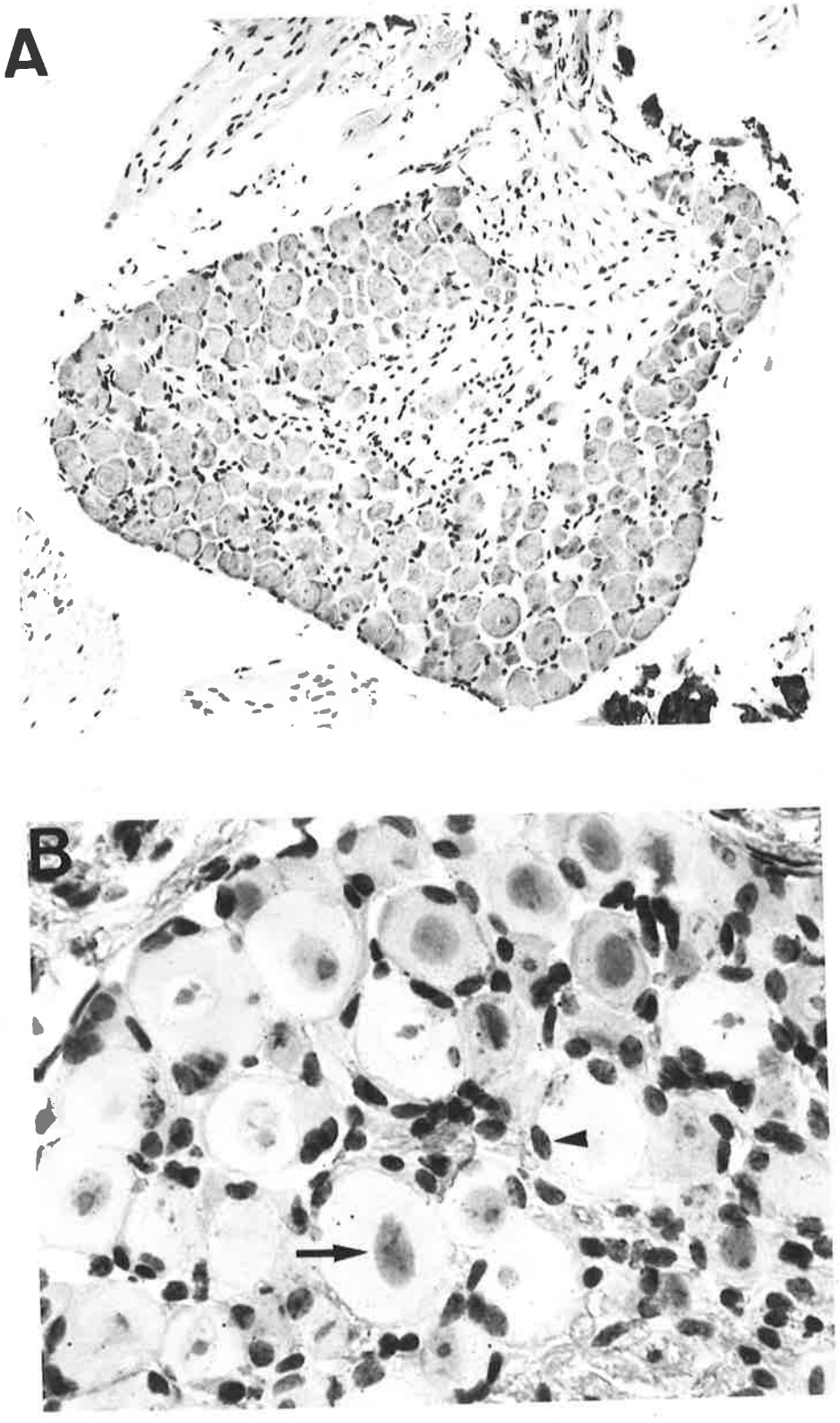
virus from the infected epithelium (Whitley, 1990).

Within dorsal root ganglia are cell bodies of primary sensory neurons, which are surrounded by a single layer of tightly packed capsular cells (Warwick & Williams, 1973) (Figure 1.2). The axons of primary sensory neurons bifurcate at a T-junction to emit the central and peripheral processes of the sensory nerve. The sensory nerve fibres from a single dorsal root ganglion innervate a segment of skin called a dermatome (Foerster, 1933; Warwick & Williams, 1973). During the course of the primary infection, HSV gains access to sensory nerve endings innervating the infected site and is transported to the associated ganglia. Virus is transported to ganglia either as intact HSV virions or viral capsids by retrograde axonal flow, at a rate of approximately 10 mm/hr (Goodpasture, 1929; Kristensson *et al.*, 1971; Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Cook and Stevens, 1973; Stevens and Cook, 1974; Stevens, 1975; Cook and Stevens, 1976; Lycke *et al.*, 1984). On reaching the ganglia virus replicates in primary sensory neurons and infectious virus can be recovered from ganglionic homogenates (Rock, 1993). Virus also reseeds the site of primary infection (Cook and Stevens, 1973). At some stage during this process latent infection is established in sensory ganglia (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Stevens, 1989).

Figure 1.2

(A) Photomicrograph (magnification x 75) of a section through a spinal ganglion of a C57BL10 mouse, showing neurons (large rounded cells) surrounded by small, flattened satellite cells and nerve fibres (top).

(B) Photomicrograph (magnification x 384) of a section through a mouse spinal ganglion. Arrowhead shows the nucleus of a satellite cell. Arrow shows a neuronal nucleus.


A
B

1.4.2 Latent infection

A hallmark of both HSV-1 and HSV-2 infection is the establishment of a life-long latent infection following the primary infection. During the period of latency (ie. between primary and recurrent infection or between periods of recurrent infection), the virus is maintained in a non-replicating state, and no infectious virus or viral proteins can be detected in latently infected hosts (Stevens, 1989). A variety of approaches, including organ culture, explant co-cultivation, and *in vivo* reactivation following neurectomy implicate sensory ganglia as the predominant site of latent HSV infection (Plummer *et al.*, 1970; Stevens and Cook, 1971; Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Knotts *et al.*, 1973; Walz *et al.*, 1974; Cook and Stevens, 1976; Warren *et al.*, 1977). Furthermore, the viral genome can be detected in sensory ganglia by Southern blot analysis (Rock and Fraser, 1983, 1985: Efstathiou *et al.*, 1986). In humans, the main sites of latency are the trigeminal ganglia and sacral ganglia, as a result of orofacial and genital infections respectively. Two lines of evidence suggest that sensory neurons are the predominant cell type harbouring latent virus. First, neurons are the first cell type in which virus or viral antigen can be detected after reactivation of latent infection (Cook *et al.*, 1974; McLennan and Darby, 1980; Kennedy *et al.*, 1983; Wroblewska *et al.*, 1989). Second, viral transcription has been detected in sensory neurons during latency (eg. Galloway *et al.*, 1979; Tenser *et al.*, 1982; Stroop *et al.*, 1984; Stevens *et al.*, 1987). In addition to sensory ganglia, virus has been recovered from autonomic ganglia including the superior cervical and vagal ganglia

(Price *et al.*, 1975; Martin *et al.*, 1977; Warren *et al.*, 1978) and the adrenal medulla (Cook and Stevens, 1976) during periods of latent infection. Extraneural latency, involving peripheral tissues such as the skin and mucous membranes, has been described both in humans and in animal models of HSV infection (Scriba, 1977, 1981; Hill *et al.*, 1980; Schimeld *et al.*, 1982; Subak-Sharpe *et al.*, 1984; Clements and Subak-Sharpe, 1988; Clements and Jamieson, 1989; O'Brien and Taylor, 1989; Pavan-Langston *et al.*, 1989). Extraneural latency remains an object of debate and its significance is uncertain. There is, however, little doubt that the nervous system, particularly sensory ganglia, plays an integral role in the pathogenesis of latent infection, yet the mechanisms involved in the establishment and maintenance of latency are poorly understood. From time to time latency may be interrupted, resulting in the production of new infectious virus and recurrent infection.

1.4.3 Recurrent infection

Reactivation of latent HSV infection may result in asymptomatic viral shedding (recurrence) or clinical disease such as lesion formation (recrudescence). Restriction endonuclease mapping of viral DNA isolated from separate episodes of recrudescence from the same person reveal identical patterns in most cases (Schmidt *et al.*, 1984). In addition, viral isolates from multiple infected ganglia from the same person appear identical (Lonsdale *et al.*, 1979) and recurrent infection occurs almost exclusively in individuals who possess neutralizing antibody to the homologous virus (Andrews & Carmichael, 1930).

Recurrent infection is due primarily to endogenous reactivation of latent virus. However, particularly in immunocompromised patients, multiple strains of the same sub-type have been detected in the same person, suggesting that exogenous re-infection with different strains of the same sub-type is possible (Heller *et al.,* 1982; Buchman *et al.,* 1979). In addition, re-infection with the same stain of HSV can occur by autoinoculation at a distant site (Nahmias *et al.*, 1970b). The frequency of exogenous re-infection is very low and does not account for the vast majority of apparent reactivations (Schmidt *et al.*, 1984; Lakeman *et al.*, 1986).

Reactivation of latent HSV is thought to occur either spontaneously or as a result of provocative stimuli, which are poorly understood. Following reactivation, virus travels centrifugally from sensory ganglia to mucocutaneous sites (Roizman, 1966). Using cultured neurons, Lycke *et al* (1984) demonstrated that virus becomes enveloped upon leaving the nucleus and infectious virus can be recovered from sensory ganglia following reactivation. Viral replication occurs at mucocutaneous sites and recrudescence is characterized by the appearance of skin vesicles or mucosal ulcers. The extent of the inflammatory response and the frequency of local lymphadenopathy is significantly less during recurrent infection compared to the primary infection (Timbury, 1993). Although recurrent infection invariably occurs in the same dermatome as the primary infection, recurrent lesions are frequently located in a different part of the dermatome with respect the site of primary infection. For

example, primary oral lesions are usually found on the inside of the mouth whereas recurrent lesions are often on the lip. In addition, although genital recrudescences often involve the vulva and surrounding skin, primary genital lesions frequently involve the uterine cervix (Corey *et al.,* 1983). The immune response controls recrudescence because immunosuppression increases the frequency and severity of lesions. However, virus appears to evade the immune system until such time as recurrent infection has begun and considerable necrosis has occurred (Simmons & Nash, 1984).

1.5 Biological and molecular aspects of latency

1.5.1 Gene expression and the establishment of latency

The extent of viral gene expression during establishment of latency has been difficult to define due to the presence of a concurrent productive infection. For this reason, studies to identify (i) genetic requirements for the establishment of latency and (ii) the point at which the molecular pathways leading to latent and productive infection diverge, have concentrated predominantly on construction and characterization of mutant viruses that interupt the replicative cycle. Methods to detect latent infection are an integral component of studies examining the establishment of latency, and explant reactivation has long been a favoured tool for this purpose. A weakness of this approach is that it cannot differentiate between failure to establish latency and failure to reactivate. Advances in molecular biological technology have allowed more definitive

markers for latency to be identified, namely: (i) latency associated transcripts (LATs), which are a prominent feature of latent infection (see 1.5.3) and (ii) HSV DNA sequences.

Temperature-sensitive mutants: Several phenotypically defined HSV temperature sensitive (*ts*) mutants that are non-permissive for viral replication at the internal temperature of mice (38.5°C) have been utilized to determine which, if any, viral genes are necessary for establishment of latency. By explant culture of ganglia at permissive temperatures (31°C-33°C) it has been shown that most *ts* mutants retain the ability to establish a reactivatable latent infection (Lofgren *et al.,* 1977; McLennan & Darby, 1980; Watson *et al.,* 1980; Al-Saadi *et al.,* 1983). Thus, *ts* mutants suggest that viral replication is not a prerequisite for the establishment of latency. However, *ts* mutant are often considered 'leaky' in that low levels of replication and spontaneous reversion to wild-type phenotype cannot be excluded. For these reasons viruses with specifically defined mutations have been favoured in recent studies.

Several genes or gene classes have been studied in detail, as follows:

α-TIF: A viral tegument protein, α-TIF, transactivates α genes and is an essential requirement for efficient initiation of the viral replicative cycle. Ace *et al* (1989) constucted a HSV-1 mutant (*in*1814), containing a 12 bp insertion in the coding region of the α-TIF gene, which synthesizes a mature but non-

functional α-TIF protein that cannot transactivate α gene expression. It was necessary to ensure that *in*1814 synthesized a complete α-TIF because this protein is an essential structural component of the virion. Viral replication cannot be detected at acute phase timepoints in ganglia or eyes of mice infected with *in*1814 by corneal scarification. However, latency is established as soon as the virus reaches trigeminal ganglia, ie. 12-24 hr after inoculation, suggesting that viral replication is not a prerequisite for the establishment of latent infection (Steiner *et al.,* 1990). Valyi-Nagy *et al* (1991) also used *in*1814 to further examine requirements for the establishment of latency with respect to expression of viral genes. The expression of α, β and γ genes and levels of viral DNA in ganglia of mice infected with *in*1814 were significantly reduced in comparison to wild type infection, yet LATs were expressed in a wild type manner. In addition, the low level gene expression and increase in viral DNA was detected only after latency had already been established, strongly implying that establishment occurred without viral gene expression or DNA replication. The fact that latency can be established in the absence of α-TIF led to the hypothesis that latent infection may be the result of failure to transport α-TIF to the nuclei of infected neurons, retarding or blocking the initiation of productive infection. α-TIF can enter the nucleus independently of viral DNA (Batterson and Roizman, 1983), and it has been suggested that it may be left behind during axonal travel due to the large distance between the site of viral entry at the periphery and the neuronal nucleus. However, Sears *et al* (1991) constructed an HSV-1 mutant containing a second copy of the α-TIF gene

under the control of an inducable foreign promoter and demonstrated that α-TIF expression did not prevent the establishment of latency. Therefore, α-TIF is unlikely to be the factor determining the establishment of latency.

Although a functional α-TIF is dispensable for the establishment of latency, the TAATGARAT motif that it recognizes to facilitate α gene transactivation may play a role in the establishment process. A cellular octamer binding protein (OCT-2) has been identified in mouse neuroblastoma cells which can repress the activity of α gene promoters and thus prevent the productive cycle of HSV replication. Therefore, if these isoforms of OCT-2 are present in neurons *in vivo*, they may prevent the initiation of replication and thereby facilitate the establishment of latency (Kemp *et al.*, 1990; Lillycrop *et al.*, 1994).

Thymidine kinase: Virally encoded thymidine kinase (TK) has been extensively studied, due to its association with viral neurovirulence. Viral TK is dispensable for viral replication *in vitro* (Dubbs and Kit, 1964) and in peripheral tissues *in vivo* (Tenser *et al.*, 1979, 1981). However, viral TK is essential for HSV replication in non-dividing cells such as neurons which contain very low levels of cellular TK (Jamieson and Subak-Sharpe, 1974; Tenser and Dunstan, 1979). Initial studies suggested that viral TK was required for the establishment of latency because TK deficient viruses failed to replicate in, or be reactivated from mouse ganglia (Price and Khan, 1981; Field and Wildy, 1978; Tenser and Dunstan, 1979; Tenser *et al.*, 1979). However, the

inability to reactivate infection does not necessarily mean that the virus cannot establish a latent infection. Rather, it may demonstrate an essential role for viral TK in reactivation, rather than in the establishment of a latent infection. A complicating factor has been a variation in characteristics of various TK deficient mutants which may, for instance, have mutations affecting other genes (eg UL24 which partially overlaps the TK gene), or leakiness of the virus with respect to TK expression. Recently, several studies have shown definitively that viral TK is not required for the establishment of latency. For instance, Sears *et al* (1985b) found that the level of TK expression and the efficiency of latency establishment do not correlate. In 1988, Ho and Mocarski used a β-galactosidase expressing TK deficient HSV and showed that mouse neuronal cells could be successfully infected in the absence of detectable viral replication. Further, TK^- HSV has also been rescued from ganglia during the latent phase by *in vitro* superinfection (Efstathiou *et al.*, 1989; Coen *et al.*, 1989). Ganglia from mice infected with TK^- HSV contain HSV DNA (Leist *et al.*, 1989; Katz *et al.*, 1990) and have been conclusively shown to express LATs (Coen *et al.*, 1989; Leist *et al.*, 1989; Tenser *et al.*, 1989; Kosz-Vnenchak *et al.*, 1990; Jacobson *et al.*, 1993). In conclusion, it appears that viral TK is not required for establishment of latency but may play a significant role in the reactivation process, by allowing virus to replicate in neurons.

Latency associated transcripts: During latency the HSV genome is transcriptionally silent with the exception of a small region encoding latency associated transcripts (LATs); which partially overlap and are antisense to the $\alpha 0$ gene. LATs are transcribed during productive and latent infection and consequently their role in the establishment of latency has been extensively examined. HSV mutants that disrupt the expression of LATs grow with normal kinetics in cultured cells and acutely infected animals and can be reactivated from latent timepoints by standard explant culture (Ho and Mocarski, 1989; Steiner *et al.*, 1989; Leib *et al.*, 1989b; Sedarati *et al.*, 1989; Block *et al.*, 1990; Natarajan *et al.*, 1991). Latent infection has also been confirmed by detection of β-galactosidase expression in neurons of mice latently infected with a LAT deficient mutant containing the *Escherichia coli lac*Z gene under LAT promoter control (Ho and Mocarski, 1989). In addition, viral DNA can be detected in ganglia during latency following infection with LAT deficient mutants, and the amount recovered is indistinguishable from that of wild-type virus (Sedarati *et al.*, 1989; Mitchell *et al.*, 1990b). However, it has recently been reported that although LATs are not an absolute requirement for the establishment of latency, they appear to promote anatomical site-dependent establishment since LAT$^-$ mutants establish latency less efficiently in trigeminal ganglia than in lumbosacral ganglia (Sawtell and Thompson, 1992).

α genes: Genes such as α4 and α0 encode transactivators that play a crucial regulatory role in the viral replicative cycle, and therefore modulation of α gene expression may influence whether productive or latent infection is established. Viruses containing mutations in various α genes have failed to identify a gene product necessary for latency establishment. Deletion mutants of α22 can successfully enter latency and be reactivated by explant cultivation (Sears *et al.*, 1985a; Meignier *et al.*, 1988), as does α47 deficient HSV (Meignier *et al.*, 1988). Leib *et al* (1989a) made 3 α0 deletion mutants that could not replicate in the eyes or ganglia of mice infected by corneal scarification and varied in their ability to reactivate by conventional explantation. The variation in the phenotypes of these 3 mutants were thought to be due to differences in the parental virus strain, location of the mutations, and/or additional mutations in other genes. This study also examined α4 and α27 null mutants that did not replicate during acute phase timepoints and did not establish a reactivatable latent infection. However, it could not be concluded whether reactivation negative latency had been established. Clements and Stow (1989) further analysed one of the α0 mutants and found that α0 was dispensable for the establishment of latency because the virus could be reactivated. Katz *et al* (1990) detected latent HSV DNA by polymerase chain reaction (PCR) in ganglia of mice infected with an α4 deletion mutant. In a separate study, an extensive evaluation of an α4 deletion mutant demonstrated the presence of LATs and HSV DNA in ganglia of mice at latent phase time points. This mutant was shown to establish latency with

extreme restriction (possibly complete absence) of viral gene expression (with the exception of LATs), yet the virus could be reactivated by superinfection with an $\alpha 4$ competent agent (Sedarati *et al.*, 1993). These results suggest strongly that establishment and maintenance of latent infection does not require expression of genes associated with productive infection.

A wild-type, virulent HSV strain (SC16) has also been utilized to investigate the establishment of latency. Using unique features of a mouse flank inoculation model, Speck and Simmons (1991) demonstrated the presence of latency (as determined by the detection of LAT^+ neurons and the ability to reactivate virus) in mouse sensory ganglia in which the acute infection had not occurred. They concluded that replication-competent HSV could establish latency without initiating productive infection. This conclusion is supported by the observation that viral antigen positive, as well as latently infected neurons, appear synchronously in spinal ganglia during the earliest stages of acute ganglionic infection with HSV strain SC16 (Speck and Simmons, 1992). Thus, there exists a large body of evidence suggesting that the pathways of lytic and latent infection can diverge at an early stage, prior to expression of any virally encoded genes, other than LATs.

1.5.2 State of the viral genome during latency

Characterization of latent viral DNA has focused predominantly on animal models of infection, but these studies have often been hampered by the relatively small amounts of HSV DNA recovered during latency. The presence of latent HSV DNA was first demonstrated by Puga *et al* (1978) who showed its existence in trigeminal ganglia and also concluded that the genome was maintained in a non-replicating, truly latent state. In 1980, Cabrera *et al* reported the presence of HSV DNA in trigeminal ganglia and brainstems of latently infected mice following corneal scarification. Significantly, only a small proportion of HSV DNA^+ brainstems could be reactivated yet almost all HSV DNA^+ trigeminal ganglia could be reactivated. The difference between latent infections in the peripheral and central nervous systems was, at the time, hypothesized to be due to the presence of incomplete or defective genomes in the central nervous system (CNS) (Cabrera *et al.*, 1980). Following this report, further studies have aimed at determining the completeness and structural configuration of latent HSV DNA by examining the abundance of specific viral restriction fragments. Initial reports using this approach suggested that linear, as well as incomplete genomes, were present in human brain samples (Fraser *et al.*, 1981), and that structural changes such as gene rearrangement or integration into cellular DNA may occur during the establishment of latency (Puga *et al.*, 1984). In contrast, Rock and Fraser (1983) published a landmark report in which they structurally analysed HSV DNA from the brains and ganglia of latently infected mice. In agreement with previous reports (Knotts *et*

al., 1973; Cabrera *et al.*, 1980; Kastrukoff *et al.*, 1981), HSV could not be reactivated from CNS material yet the majority of latent DNA was found in this tissue. Significantly, latent genomes in mouse brain appeared to be complete since all HSV-1 specific probes used detected fragments identical in size to those of virion DNA. Thus, the inability to reactivate was not due to the presence of incomplete or defective latent genomes. In addition, a probe specific for the junction and terminal fragments of HSV-1 failed to detect the termini of the viral genome during latency, indicating a clear structural difference between viral DNA isolated from acutely and latently infected mice. Specifically, the termini of the viral genome were undetectable in both brain and ganglia during latency, strongly arguing that the HSV genome was 'endless' and existed either in a circular, concatemeric or integrated form, and not as unit length linear DNA. These conclusions were confirmed by Efstathiou *et al* (1986) who demonstrated the presence of 'endless' HSV DNA in brain and ganglionic tissue of latently infected mice, as well as human trigeminal ganglia. The 'endless' genomes were present in all 4 isomeric configurations and were both quantitatively and qualitatively stable during latency. A detailed analysis of the relative proportions of viral junction and terminal regions by Rock and Fraser (1985) showed that the lack of termini in latent HSV-1 DNA was due to the joining of termini to form a fragment equivalent to the virion DNA junction fragment. Therefore, the loss of the termini was not due to the deletion or integration via these regions into cellular DNA. This suggests that at least the majority of latent DNA exists in a circular

or concatemeric form although integration via other viral sequences or integration of a large concatemer could not be excluded. Mellerick and Fraser (1987) subsequently analysed latent HSV DNA by density gradient centrifugation and showed that the majority of the viral DNA recovered during latency could be separated from host chromosomal DNA and therefore persisted in a non-integrated state. However, because a small amount of HSV DNA could not be separated from host DNA, the possibility still exists that a small fraction of latent viral DNA is integrated.

Regardless of its structural configuration, micrococcal nuclease digestion has demonstrated that HSV-1 DNA from the brains of latently infected mice is packaged with histones in a nucleosomal structure similar to that of cellular chromatin (Deshmane and Fraser, 1989). All of the major regions of the viral genome, including the LAT locus were found to be associated with nucleosomes, and it has been proposed that this chromatin structure may play an important role in the regulation of viral gene expression during latency.

Methylation of DNA has been recognized as an effective repressor of transcriptional activity and is associated with differential regulation of many cellular genes in different cell types (Yisraeli and Szyf, 1984; Cedar, 1988). Thus, latent HSV DNA may be methylated so as to render the viral genome inactive during latency. Although hypermethylation of the HSV genome in an *in vitro* latency model has been described (Youssoufian *et al.*, 1982), viral

DNA from the CNS of latently infected mice is not extensively methylated (Dressler *et al.*, 1987). However, treatment of ganglionic and CNS tissue from latently infected animals with demethylating agents enhances virus reactivation (Stephanopoulos *et al.*, 1988; Bernstein and Kappes, 1988; Whitby *et al.*, 1988). The extent of methylation of latent HSV DNA in the peripheral nervous system (PNS), and the significance of methylation in terms of repression of the viral genome are yet to be elucidated.

Assessment of the amount of HSV DNA per latently infected cell has been difficult due to the inability to directly detect viral DNA *in situ*. Instead, indirect methods of quantification have been utilized by several research groups. Early studies using hybridization kinetics analysis of latently infected mouse brain and ganglia yielded estimates of 0.03-0.11 viral genome copies per cell (Puga *et al.*, 1978; Cabrera *et al.*, 1980). Rock and Fraser (1983) analysed the brains of latently infected mice by Southern blot hybridization and found 0.015-0.15 copies per cell. Efstathiou *et al* (1986) used Southern blot hybridization to estimate the amount of viral DNA in brainstems (0.26 copies per cell), spinal cords (0.19 copies per cell) and cervical ganglia (0.78 copies per cell) of latently infected mice. They also detected 0.01-0.1 HSV genomes/cell in human trigeminal ganglia. Quantitative polymerase chain reaction (PCR) has also been applied to latently infected mouse ganglia, resulting in estimates of 0.3-3 viral genome copies per cell (Katz *et al.*, 1990; Sawtell and Thompson, 1992). Thus, latent viral DNA in mouse nervous

system tissues or human ganglia is present in the range of 0.01-3 copies per cell. Ramakrishnan *et al* (1994) used competitive quantitative PCR analysis to detect HSV DNA and LATs in brains of rats infected with a highly attenuated HSV-1 mutant. In this study it was estimated that there were 2.6 x 10^5 genome equivalents per 10 μm hippocampal section and that there were approximately 15 LAT molecules per viral genome equivalent. This study did not attempt to estimate the number of viral genomes per cell.

Calculation of the number of HSV genomes per latently infected cell has been based upon the following: (i) the site of latent virus is most likely to be neurons (Cook *et al.*, 1974; McLennan and Darby, 1980), (ii) approximately 10% of cells in mouse dorsal root ganglia are neurons (Walz *et al.*, 1976) and (iii), typically about 0.1-1% of neurons from enzymically dissociated latently infected ganglia yield reactivatable virus (Walz *et al.*, 1976; Kennedy *et al.*, 1983; Nicholls and Blyth, 1989). Taking all of these observations into account, there would be a minimum of approximately 10 and a maximum of approximately 30000 HSV genomes per latently infected neuron. This estimate is based on the assumptions that viral DNA does not persist in cells other than neurons, and that all latent genomes exist in a reactivatable form. Given that, in CNS material at least, HSV DNA reactivates with poor efficiency, it is by no means certain that reactivation (especially by explant cultivation) betrays the presence of all latent genomes. Similarly, the use of LAT positivity as a marker to enumerate latently infected neurons assumes that all latently infected

cells contain viral genomes which actively transcribe detectable amounts of LATs. Using LATs rather than reactivation as a marker of latency, Rødahl and Stevens (1992) estimated there to be 174 latent viral genomes per LAT^+ neuron in superior cervical ganglia of mice latently infected with HSV-1 strain KOS(M). They also reported that LATs accumulated differently in sensory and autonomic ganglia. Although autonomic ganglia harboured substantial amounts of viral DNA, LAT^+ neurons were not detected. Thus, some neurons may harbour a latent infection in the absence of LAT expression. Margolis *et al* (1992) estimated there to be 120 HSV genome copies per LAT expressing neuron in ganglia of mice latently infected with HSV-1 strain KOS(M) by bilateral sciatic inoculation. Further, they provided evidence that, in their model at least, the latent viral DNA was not a result of DNA amplification during the establishment of latency.

1.5.3 Transcription during latency

1.5.3.1 Detection of latency associated transcripts

Latent HSV infection is commonly referred to as the non-productive phase since neither infectious virus nor viral proteins have been conclusively demonstrated during this period *in vivo*. However, the viral genome is not completely transcriptionally silent during latency. Several early reports demonstrated the presence of HSV specific RNA transcripts from various regions of the viral genome in ganglionic tissues from infected animals and humans, (Galloway *et al.*, 1979, 1982; Tenser *et al.*, 1982; Stroop *et al.*, 1984)

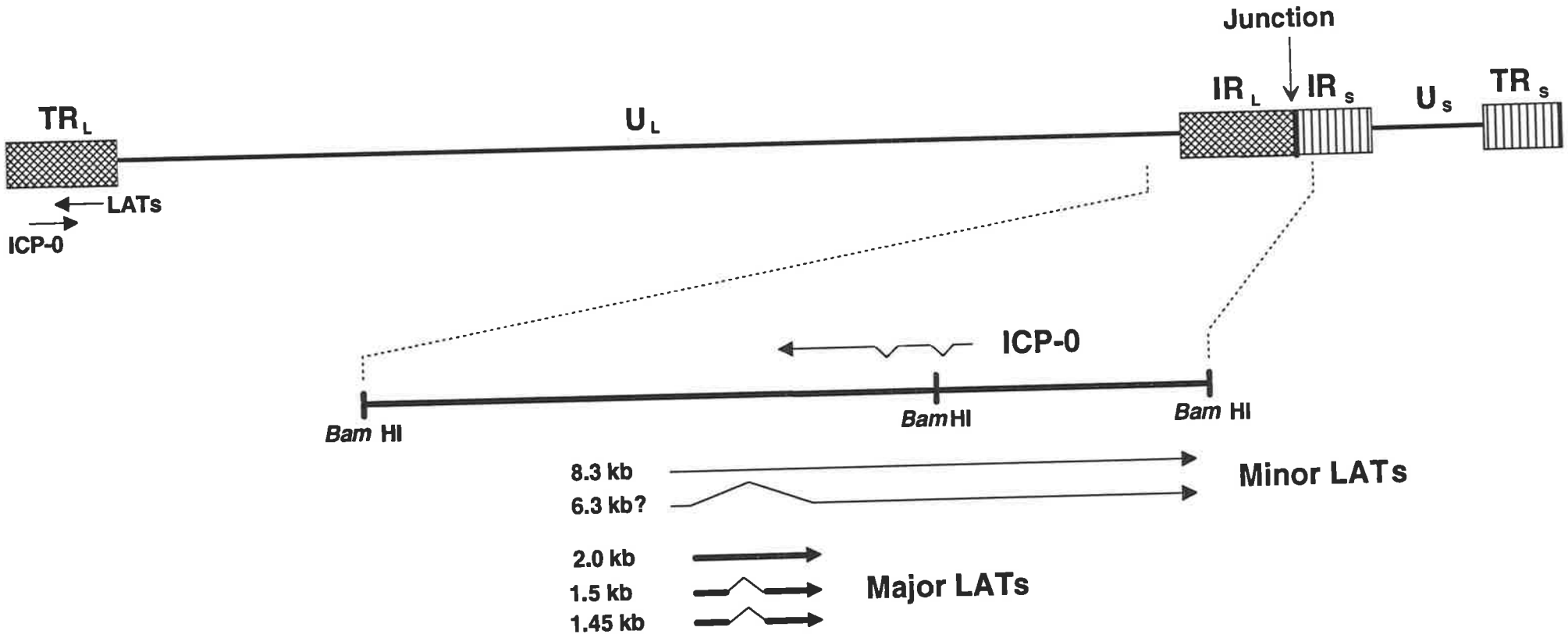
but some of these studies were complicated by possible reactivation of virus during the experiment. Puga and Notkins (1987) showed that both the IR_L and TR_L regions of the HSV genome were transcriptionally active during latency and that these transcripts originated from DNA sequences in the region of the $\alpha 0$ gene (Figure 1.3). *In situ* hybridization has demonstrated that these latency associated transcripts (LATs) localize to neuronal nuclei during latency, but are found in both the nucleus and cytoplasm during acute infection (Stevens *et al.*, 1987; Deatly *et al.*, 1987). Significantly, both northern blot and *in situ* hybridization studies have conclusively shown that LATs partially overlap the 3' end of $\alpha 0$ mRNA but are transcribed in the opposite direction, and therefore are antisense to $\alpha 0$ mRNA (Rock *et al.*, 1987b; Stevens *et al.*, 1987; Croen *et al.*, 1987; Spivak and Fraser, 1987; Krause *et al.*, 1988). Spivak and Fraser (1988b) utilized a deletion mutant to demonstrate that the gene encoding LATs is not an α gene. Neither is it a β or γ gene because these gene classes require α gene expression for their activation, yet LAT expression occurs independently of α gene activity. Thus, the authors proposed that LATs constitute a new class of genes which they termed lambda (λ).

1.5.3.2 Characterization of LATs

Depending on the viral strain used, two or three collinear LATs, with approximate sizes of 1.45 kb, 1.5 kb and 2.0 kb, have been detected in RNA extracted from latently infected ganglionic tissue (Rock *et al.*, 1987b; Spivak and Fraser, 1987, 1988a, b; Krause *et al.*, 1988; Wagner *et al.*, 1988b;

Figure 1.3

Map of the HSV-1 genome showing the region from which LATs are transcribed. Major LATs (approx. 1.45 to 2.0 kb) are shown as thick arrows. The minor LATs (approx. 6.3 to 8.3 kb), are shown as thin arrows. LAT sequences overlap, and are transcribed in the opposite direction to the $\alpha 0$ gene, which encodes ICP-0 (thin arrow). (L: long segment; S: short segment; TR_L: long terminal repeat region; U_L: long unique region; IR_L: long internal repeat region; IR_S: short internal repeat region; U_S: short unique region; TR_S: short terminal repeat region).


Junction
IR_L
IR_S
U_S
TR_S
TR_L
U_L
LATs
ICP-0
ICP-0
Bam HI
BamHI
Bam HI
8.3 kb
6.3 kb?
Minor LATs
2.0 kb
1.5 kb
1.45 kb
Major LATs

Wechsler *et al.*, 1988). These are commonly referred to as major LATs (Figure 1.3). Major LATs are devoid of detectable polyadenylation and are located mostly in the nucleus of latently infected neurons (Rock *et al.*, 1987b; Spivak and Fraser, 1987; Wagner *et al.*, 1988b). The most abundant transcript is the 2.0 kb species which, depending on viral strain and host, makes up between 50% and 90% of the total latent RNA (Wagner *et al.*, 1988b). The smaller major LAT species are thought to be derived from the 2.0 kb RNA by splicing, which may involve neuron-specific factors (Deatly *et al.*, 1988; Wechsler *et al.*, 1988; Wagner *et al.*, 1988b; Mitchell *et al.*, 1990b; Spivak *et al.*, 1991). The putative splicing event giving rise to the 1.45 kb and 1.5 kb species would preserve one of the two open reading frames in this region (Wechsler *et al.*, 1988; Wagner *et al.*, 1988b). Although major LATs predominate in latently infected neuronal cells, the 2.0 kb species can also be detected, albeit at very low levels, in lytically infected cultured cells, acutely infected peripheral tissues and acutely infected ganglia (Spivak and Fraser, 1988a; Steiner *et al.*, 1988; Wagner *et al.*, 1988a). Major LATs have been detected in ganglia from seropositive humans indicating that they are not an artifact of animal latency models (Croen *et al.*, 1987; Gordon *et al.*, 1988; Krause *et al.*, 1988, Steiner *et al.*, 1988).

Several studies have been undertaken to identify the promoter responsible for expression of LATs. Unlike most eukaryotic genes, the region immediately upstream of the nominal start site of major LATs is devoid of any TATA

consensus elements. Surprisingly, the nearest polymerase II promoter consensus sequence lies approximately 700 bp upstream of the 5' end of the 2.0 kb major LAT (Wagner *et al.*, 1988a; Wechsler *et al.*, 1989). This region contains a TATA box, a potential CAAT box and three Sp1 binding sites, which strongly suggest that it is a transcriptional promoter (Jones and Tijan, 1985; Kadonaga *et al.*, 1987). The TATA box promoter regions of HSV-1 and HSV-2 display a high degree of homology (Krause *et al.*, 1991). Transient chloramphenicol acetyltransferase (CAT) assays have shown that this region confers promoter activity in cells of both neuronal and non-neuronal origin (Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990, 1991), and studies using HSV mutants support its authenticity as a LAT promoter (Javier *et al.*, 1988; Dobson *et al.*, 1989; Leib *et al.*, 1989b; Steiner *et al.*, 1989). In addition, other potential regulatory elements which have the capacity to bind various viral and cellular factors have been identified in the promoter region (Batchelor and O'Hare, 1992; Leib *et al.*, 1991; Zwaagstra *et al.*, 1991). In particular, the promoter is repressed by ICP-4, and this repression is conferred by a 55 bp region downstream of the TATA box (Batchelor and O'Hare, 1990). This finding is consistent with the observation that LAT expression is lowest during times of viral replication and that the amount of LATs decreases following reactivation from latency (Spivak and Fraser, 1988a). The putative LAT promoter has been mapped to a region of 140 bp, from approximately -800 to -660 (Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990, 1991).

The unusual relationship between major LATs and their promoter has led to the hypothesis that major LATs are stable introns derived from a low abundance larger transcript that originates from this promoter (Dobson *et al.*, 1989; Zwaagstra *et al.*, 1990; Farrell *et al.*, 1991). This is supported by sequence analysis of regions surrounding major LATs which has revealed the presence of consensus splice donor and acceptor sites (Wagner *et al.*, 1988b). These sites have been shown to be active during latency (Farrell *et al.*, 1991). Furthermore, it has been reported that regions of the viral genome flanking the major LATs are transcriptionally active during latency (Rock *et al.*, 1987b; Deatly *et al.*, 1987; Mitchell *et al.*, 1990b). Mitchell *et al* (1990b) reported the presence of viral transcripts from an approximately 8.3 kb region in latently infected ganglia, and northern blot analysis of RNA from productively infected cells has demonstrated a very weak poly $(A)^+$ 8.3 kb LAT species (Dobson *et al.*, 1989; Mitchell *et al.*, 1990b; Zwaagstra *et al.*, 1990; Devi-Rao *et al.*, 1991). In addition this LAT species has been detected at low abundance in RNA from latently infected rabbit ganglia (Zwaagstra *et al.*, 1990). Owing to their low abundance, transcripts spanning the abundant major LATs are commonly referred to to as minor LATs (mLATs). Thus, mLATs may represent an unstable primary transcript which is subsequently processed to produce the more stable 1.45 kb, 1.5 kb and 2.0 kb poly $(A)^-$ major LATs (Figure 1.3). At present, however, the 6-7 kb spliced poly $(A)^+$ product of this processing has not been observed.

There is, however, data that is not consistent with there being a single LAT promoter located far upstream of a spliced major LAT species. A *lac*Z reporter gene inserted into the LAT region at position +137 relative to the 5' end of major LAT displayed punctate staining during latency but was not active during productive infection (Ho and Mocarski, 1989). RNase mapping demonstrated that these transcripts initiated near the 5' end of major LATs rather than at the putative LAT promoter, suggesting that trancription may initiate from a cryptic promoter. Recently, a second putative LAT promoter (LAP2) has been identified and partially characterized (Goins *et al.*, 1994). Transient CAT assays were employed to detect a latency-active promoter within the downstream region of the previously reported LAT promoter (LAP1). In contrast to LAP1, this promoter lacks a TATA box. However, LAP2 possesses *cis*-acting regulatory elements and other features observed within eukaryotic housekeeping gene promoters, and was down regulated by the viral transcriptional transactivators ICP-0 and ICP-4. LAP2 is active in neuronal cell cultures infected with a non-replicating recombinant HSV, although its activity is 5-10 fold less than that of LAP1. It is possible that LAP2 does not act as an independent promoter but rather is part of a complex promoter/regulatory region in conjunction with LAP1.

1.5.3.3 *LATs in other herpesviruses*

LATs similiar to those of HSV-1 have also been detected in ganglia latently infected with HSV-2 (Suzak & Martin, 1989; Mitchell *et al,* 1990a; Krause *et al,* 1991; Tenser *et al,* 1991). HSV-2 LAT comprises a single 2-3 kb major species which overlaps and is antisense to the 3' end of HSV-2 α0 mRNA (Mitchell *et al.*, 1990a). In addition, DNA sequences corresponding to the LAT region of HSV-1 are present in other herpesviruses including equine herpesvirus 1 (EHV-1); varicella zoster virus (VZV), pseudorabies virus (PSV) and bovine herpesvirus 1 (BHV-1). PSV LATs originating from a region overlapping an IE gene have been detected in latently infected ganglia. There are poly $(A)^+$ and poly$(A)^-$ transcripts from this region with approximate sizes of 4.5-5.5kb and 1.0-2.0kb (Cheung, 1989; Lokensgard *et al,* 1990; Priola *et al,* 1990) and like HSV-1 LATs they are thought to be derived from a larger, unstable primary transcript (Cheung, 1991; Priola & Stevens, 1991). Transcription from the BHV-1 genome has been demonstrated in neurons of latently infected trigeminal ganglia. These predominantly nuclear transcripts, ranging in size from 0.77-1.16kb, map to a region overlapping and complementary to an IE gene (Rock *et al,* 1987a; Kutish *et al.*, 1990). In VZV infection, there may be a broader pattern of latent transcription, with no less than 5 distinct regions of the genome encoding latent transcripts (Croen *et al,* 1988). Therefore, it appears that LAT elements have been conserved amongst several herpesviruses, suggesting that they encode a significant function.

1.5.3.4 *Potential functions of LATs*

Major LATs may encode a protein but several observations do not support this hypothesis. Although the sequence of HSV-1 LATs contains at least two open reading frames (ORF), one of which is conserved between HSV-1 strains (Wagner *et al.*, 1988a; Wechsler *et al.*, 1989; Spivak *et al.*, 1991), there is little sequence conservation between HSV-1 and HSV-2 (Mitchell *et al.*, 1990a) or between HSV-1 and PRV (Cheung, 1989; 1990). Also, the LAT region outside $\alpha 0$ coding sequences does not exhibit triplet-based periodicity in base frequencies which is characteristic of most HSV protein coding DNA regions, and shows no conservation with the equivalent HSV-2 region, which has a different distribution of stop codons. In addition, there are frameshift differences in LAT sequences of different strains of HSV-1 (Perry and McGeoch, 1988; McGeoch *et al.*, 1991). Major LATs are not extensively polyadenylated (Rock *et al.*, 1987b; Spivak and Fraser, 1987; Wagner *et al.*, 1988b) and their localization to the nucleus of neurons during latency suggest that they do not encode a protein. Further, antibodies raised against synthetic peptides constructed from the DNA sequence of the LAT gene have failed to detect a protein in latently infected ganglia (Wagner *et al.*, 1988a; Wechsler *et al.*, 1989). Although no latent HSV proteins have been detected *in vivo*, the low abundance 8.3 kb minor LAT species is polyadenylated, and may therefore encode an as yet unidentified protein product. Doerig *et al* (1991a) have identified a latency associated antigen (LAA) in neurons latently infected *in vitro*, using an antiserum against a bacterially expressed fusion protein

containing part of an ORF within the abundant 2.0 kb LAT species. However, LAA has a molecular weight of approximately 80 kDa, yet the largest ORF of HSV-1 LAT would encode a predicted protein of only 35 kDa (Wechsler *et al.*, 1989; McGeoch *et al.*, 1991; Spivak *et al.*, 1991).

The nuclear location of LATs and their overlap with the $\alpha 0$ gene, suggests that they may function as an antisense repressor of $\alpha 0$ gene expression (Stevens *et al.*, 1987). Binding of antisense RNA derived from the opposite strand to the mRNA causing inhibition of expression of a particular gene has been described both in prokaryotes (Simons and Kleckner, 1983) and eukaryotes (Izant and Weintraub, 1984). Farrell *et al* (1991) reported the ability of LATs to inhibit $\alpha 0$ mRNA expression in transient infection assays. If $\alpha 0$ mRNA transcription was physically repressed by binding of LAT RNA *in vivo*, then α gene-dependent transactivation of β and γ genes which depend on α gene transcription, would be blocked. This mechanism could regulate two separate aspects of HSV infection. First, antisense repression could promote the establishment of latency by shutting down the viral replicative cycle. This hypothesis is not supported by experimental evidence which has found that LAT deficient mutants can establish and maintain a latent infection (Ho and Mocarski, 1989; Steiner *et al.*, 1989; Leib *et al.*, 1989b; Sedarati *et al.*, 1989; Block *et al.*, 1990; Mitchell *et al.*, 1990b). There has been, however, a report of HSV-1 LATs playing a role in the establishment of latency in trigeminal ganglia but not in lumbosacral ganglia, suggesting a possible anatomical-

dependent function for LATs in the establishment process (Sawtell and Thompson, 1992). Second, antisense repression of $\alpha 0$ may prevent reactivation. Expression of $\alpha 0$ may be required for reactivation because $\alpha 0$ deficient mutant viruses establish latency but fail to reactivate (Leib *et al.*, 1989a). Thus, LATs may prevent reactivation by repressing any bursts of $\alpha 0$ mRNA transcription during latency, thereby inhibiting the synthesis of the $\alpha 0$ gene product. If LATs did act in this manner, LAT deficient mutants would be expected to reactivate with greater efficiency than wild-type virus. On the contrary, LAT mutants often display a reduced ability to reactivate (see below), strongly suggesting that LATs do not function as an antisense repressor. It has therefore been hypothesized that the accumulation of LATs may promote rather than hinder reactivation by providing a pool of transcripts that are ready for immediate use when conditions arise that favour the initiation of HSV replication (Steiner *et al.*, 1989). Numerous viruses defective in LAT production have been shown to establish latency. In ganglionic explants, reactivation of some, but not all, LAT mutants is impaired. Differences in the capacity to reactivate may be related to the location of the mutation affecting LAT expression. Viruses with mutations in the coding region of the LAT gene, but which retain the putative LAT promoter and continue to produce at least some of the LAT gene product, have been shown to reactivate with normal kinetics and efficiency compared with parental or revertant viral strains (Ho and Mocarski, 1989; Izumi *et al.*, 1989; Sedarati *et al.*, 1989; Dobson *et al.*, 1989; Block *et al.*, 1990). There is at least one report of a LAT null mutant

with part of the promoter region deleted that also reactivates normally from mice following footpad inoculation (Javier *et al.*, 1988). However, in general, HSV mutants that have the entire LAT promoter region deleted display slower kinetics and/or reduced frequency of reactivation (Steiner *et al.*, 1989; Leib *et al.*, 1989b; Mitchell *et al.*, 1990b; Hill *et al.*, 1990; Block *et al.*, 1993).

The efficiency of reactivation of LAT mutants has also been studied *in vivo*. Experiments by Hill *et al* (1990) and Trousdale *et al* (1991) showed that a LAT null mutant reactivated poorly from latently infected rabbits after induction of reactivation by epinephrine iontophoresis. Significantly, spontaneous reactivation (a feature of rabbit, but not mouse latency models) and reactivation by explant cultivation, occurred with wild type kinetics (Hill *et al.*, 1990). Therefore, it appears that there are differences between induced and spontaneous reactivation *in vivo*, and LATs may facilitate the former process, perhaps by maintaining the viral genome in a physical state that enables rapid and efficient reactivation. By remaining transcriptionally active (to some degree at least), the LAT region would be protected from nucleosome binding, which is a feature of latent HSV DNA (Deshmane and Fraser, 1989). The LAT locus and perhaps adjacent α gene promoters would therefore be accessible to transcription factor binding which is assumed to be necessary for the reactivation process.

Many physiological and experimental stimuli that trigger viral reactivation also have the potential to influence levels of intracellular cyclic nucleotides such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Leib *et al* (1991) identified a functional cAMP response element (CRE) in the promoter region of HSV-1. They found that the LAT CRE is responsive to a variety of cAMP modulators in CAT assays and is also responsible for high basal level activity of the LAT promoter in cells of neuronal origin. In addition, it was demonstrated that cAMP analogues accelerated reactivation of wild-type HSV but did not accelerate reactivation of an HSV mutant deleted for the LAT promoter region and 1015 bp of LAT. This finding is consistent with the report of Hill *et al* (1990) who demonstrated inefficient reactivation of a LAT-deleted virus following iontophoresis of epinephrine, a potent cAMP agonist, in a rabbit ocular model. These results suggest that LATs function in a stimulatory manner on reactivation and that overexpression of LATs induced by cAMP can lead to accelerated reactivation. Therefore, one potential model of reactivation is that intracellular levels of cAMP increase in response to either cellular or viral stimuli, leading to up-regulation of the LAT promoter which then stimulates reactivation via a function encoded by the LATs.

In summary, it has not yet been possible to attribute conclusively a function to the LATs, but, in view of the fact that all LAT mutants retain the ability to reactivate, it appears certain that they are not an absolute requirement for the establishment and maintenance of latency or reactivation.

1.5.4 *In vitro* models of HSV latency

Although animal models are widely utilized and play a pivotal role in the study of latency, they also present researchers with several significant problems. After infection, only a small percentage of neurons in experimental animals become latently infected, meaning that examination of many aspects of latency often require large numbers of (expensive) animals and extremely sensitive molecular procedures. Analyses are complicated further by the heterogenicity of neural tissues. For these reasons considerable effort has been put into developing *in vitro* models of HSV latency, but progress has been difficult because neurons become lytically rather than latently infected when exposed to HSV *in vitro*. To overcome this problem, methods have been devised to arrest the viral replicative cycle in homogenous cell cultures, thereby producing a non-lytic infection resembling latency.

It has been demonstrated that nerve growth factor (NGF) dependent neurons are the predominant sites of HSV-1 latency and that NGF is required by sympathetic and sensory neurons to survive and maintain normal function (Thoenen and Barde, 1980). With this in mind, Wilcox and Johnson (1987)

succeeded in producing long term, non-lytic HSV infection of neurons *in vitro* using an NGF-dependent latency model. A significant percentage of cultured primary sympathetic neurons survive infection and show no evidence of cytopathic effect (CPE) when inoculated with HSV-1 at low multiplicities of infection (0.03-0.5 pfu/cell). During this time, viral transcription is limited to the region encoding the LATs (Doerig *et al.*, 1991b). Interestingly, unlike latency *in vivo,* a latency associated viral antigen (LAA), encoded by a region corresponding to the LATs has also been detected (Doerig *et al.*, 1991a). Deprivation of NGF from the culture medium results in the production of infectious virus from a proportion of latently infected cultures, indicating the presence of intact HSV genomes (Wilcox and Johnson, 1987, 1988; Wilcox *et al.*, 1990). The fact that NGF deprivation reactivates latent HSV is consistent with the observation that HSV can be reactivated *in vivo* by neurectomy (Carton and Kilbourne, 1952; Walz *et al.*, 1974; Price and Schmitz, 1978).

A different approach to study latency *in vitro* is the use of cell lines capable of indefinite growth in culture, rather than neurons. Treatment of infected cells with various inhibitors of viral growth has been shown to lead to the establishment of infection that is devoid of detectable viral replication for extended periods of time. O'Neill *et al* (1972) described a system in which human embryonic lung cells were treated with the viral inhibitor, cytosine arabinoside, prior to infection with HSV-2. Cells showed no evidence of CPE and production of infectious virus was halted for 3-4 weeks. When the

inhibitor was removed, infectious virus reappeared and the cells were killed. Other models of latency have been established *in vitro* by treatment of virus infected human cells with inhibitors such as cycloheximide, followed by maintenance of latency by incubation at supraoptimal temperatures (eg. 40.5^{0}C) in the absence of the viral inhibitor (Colberg-Poly *et al.*, 1979; Wigdahl *et al.*, 1981, 1982; Shiraki and Rapp, 1986). Stimuli for reactivation include temperature reduction to 37^{0}C (Wigdahl *et al.*, 1981) and superinfection with human cytomegalovirus (HCMV) (Colberg-Poly *et al.*, 1979, 1981; Shiraki and Rapp, 1986). Structural analysis of latent viral DNA from one such model revealed the presence of viral genomes in a predominantly linear configuration (Wigdahl *et al.*, 1984), which is unlike that that found in latently infected animals and humans (Rock and Fraser, 1983; Efstathiou *et al.*, 1986). In addition, viral proteins α0 and ICP8 (Shiraki and Rapp, 1989) and RNA transcripts from the unique region of the viral genome (Scheck *et al.*, 1989) have been detected in this type of model, in stark contrast to latency *in vivo*. Russell and Preston (1986) have refined the system by establishing latent infection with HSV-2 in cultured human fetal lung cells at elevated temperature (42^{0}C), without the addition of replication inhibitors. After establishment, cultures could be returned to 37^{0}C without ensuing virus replication and reactivation could be initiated by superinfection with HCMV. In this model system, the presence of HSV-1 ICP0 stimulates virus reactivation (Russell *et al.*, 1987; Harris *et al.*, 1989). Further, viral DNA is confined to the nucleus of infected cells and exists in a non-linear configuration, resembling

the structure of latent HSV DNA recovered from latently infected ganglia (Preston and Russell, 1991).

The significance of neurons to HSV latency is without question and therefore several groups have concentrated on establishing *in vitro* latency models using proliferating cells of neuronal origin. A variety of such cell lines have been shown to be relatively resistant to lytic HSV replication (Adler *et al.*, 1978; Doller *et al.*, 1979; Vahlne and Lycke, 1977, 1978), although establishment of long term latent infection in these cells has been unsuccessful. Utilizing antiviral agents, Nilheden *et al* (1985) reported establishment of HSV-1 latency in a mouse neuroblastoma cell line. Replication of HSV-1 could be initiated by superinfection of the cultures with HSV-2. Repeated passage of the cell line eliminated this latent infection, suggesting that viral DNA was not replicated as the cells divided. LATs were not detected in this cell line at any stage of the experiment (Kemp and Latchman, 1989).

In summary, despite considerable effort, no single model of *in vitro* latent infection successfully mimics all major features of *in vivo* latency, with respect to the structure and transcriptional activity of the viral genome. Therefore, in the immediate future, animal models are likely to remain a vital component of latency research.

1.6 Aim of project

The point at which the molecular pathways leading to productive and latent infection, and the related issue of whether viral DNA is amplified during establishment of latency, have been objects of speculation for many years. HSV deletion mutants unable to express any genes associated with productive infection retain the ability to establish latent infections and, under these conditions, the amount of viral DNA is greatly reduced and analysis has been problematic. These data imply that the pathways leading to productive and latent infection diverge at a very early stage before amplification of viral DNA (see 1.5.1). Paradoxically, ganglia removed from experimental animals inoculated with virulent virus and HSV seropositive humans, contain a strikingly large number of viral genomes (see 1.5.2). These data suggest that latently infected cells each contain hundreds of HSV DNA molecules implying, in contrast to studies using replication defective mutants, that HSV DNA is amplified during or after establishment of latency. Therefore, replication defective mutants may not accurately reflect the behaviour of replication competent virus during the establishment phase.

The specific aims of this study were to (i) determine whether latent HSV genomes are a result of viral DNA amplification in the PNS during the establishment phase and (ii), investigate the relationship between HSV DNA copy number and viral transcriptional activity during latent infection of the

PNS. In order to map the distribution of viral nucleic acid sequences in latently infected sensory ganglia, experiments were undertaken using a mouse model that makes novel use of the segmental sensory innervation of flank skin.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Virus stocks

Experiments were performed with HSV-1 strain SC16 and/or TKDM21. Strain SC16 is a well characterized human oral isolate of HSV-1 with a low passage history (Hill *et al.*, 1975). It is moderately neurovirulent after cutaneous inoculation of mice and produces a transient phase of productive infection (Harbour *et al.*, 1981; Tullo *et al.*, 1982; Simmons and Nash, 1984; Simmons and La Vista, 1989; Simmons, 1989; Speck and Simmons, 1991, 1992), followed by a stable latent infection that is indistinguishable at the molecular level from that seen in humans (Efstathiou *et al.*, 1986).

TKDM21 is a well characterized, genetically engineered recombinant virus which contains an 816 bp *Nru*I-*Sma*I deletion within the thymidine kinase (TK) coding region of HSV-1 strain SC16 (Efstathiou *et al.*, 1989). TKDM21 does not exhibit any TK activity, grows normally in cell culture and has the same non-syncytial plaque morphology as the parental SC16 virus. It replicates slightly less efficiency than SC16 in ears of mice and infectious virus cannot be detected during acute phase times either in ganglia, brainstem or spinal cord. However, latent infection is established because virus can be reactivated by superinfection with wild-type virus (Efstathiou *et al.*, 1989).

Working stocks of SC16 and TKDM21 consisted of Vero cells sonically disrupted 48 hours after infection at low multiplicity, 0.1 pfu/cell with sub-master virus stocks. Stocks typically contained $5x10^9$ pfu/ml and were stored in 0.2 ml aliquots at -70^0C until required. Master and sub-master stocks were stored in liquid nitrogen.

2.1.2 Mice

Female C57BL10 mice were obtained from the Specific Pathogen-Free facility at the Animal Resource Centre, Perth, Western Australia. Genetic authenticity was tested and confirmed by the supplier on a 6 monthly basis. All mice were used at greater than 8 weeks of age.

2.1.3 Plasmids

pBAZ-1 was constructed by inserting an 875 bp *Pst*I-*Pst*I fragment of the TK gene of HSV-1 strain F into Bluescribe M13$^-$ (Stratagene Cloning Systems) (Figure 2.1). Plasmid construction and propagation was carried out using standard procedures (Maniatis *et al.*, 1982).

pBS-0 and pSLAT-4 were gifts from S Efstathiou, Cambridge University, UK. pBS-0 contains a 2557 bp *Bam*HI-*Sal*I fragment of HSV-1 strain KOS, spanning map units 0.79 to 0.807 cloned into Bluescribe M13$^-$ (Figure 2.2). Transcripts from the T7 promoter of pBS-0 are complementary to major and minor LATs. Transcripts from the T3 promoter are complementary to α0

Figure 2.1

Map of the HSV-1 genome showing the positions of cloned viral fragments used as probes in blot hybridization experiments. Box 1 shows the position of an 875 bp *Pst*I-*Pst*I fragment of the TK gene contained within the *Bam*HI-Q fragment (Q) of the genome, used to construct pBAZ-1. Box 2 shows the position of a 10.1 kb *Bam*HI-B fragment (B) of the genome used to construct pSB-6. Box 3 shows the 2.5 kb junction-specific region used to construct pBKSP1, consisting of the *Bam*HI-K fragment (K) from which has been deleted a central 3.5 *Sac*I fragment. The junction fragment K (5.9 kb) consists of the fusion of the two *Bam*HI terminal fragments P (3.6 kb) and S (2.9 kb). The repeated regions of the long and short segments of the genome are shown as hatched and lined boxes, respectively.

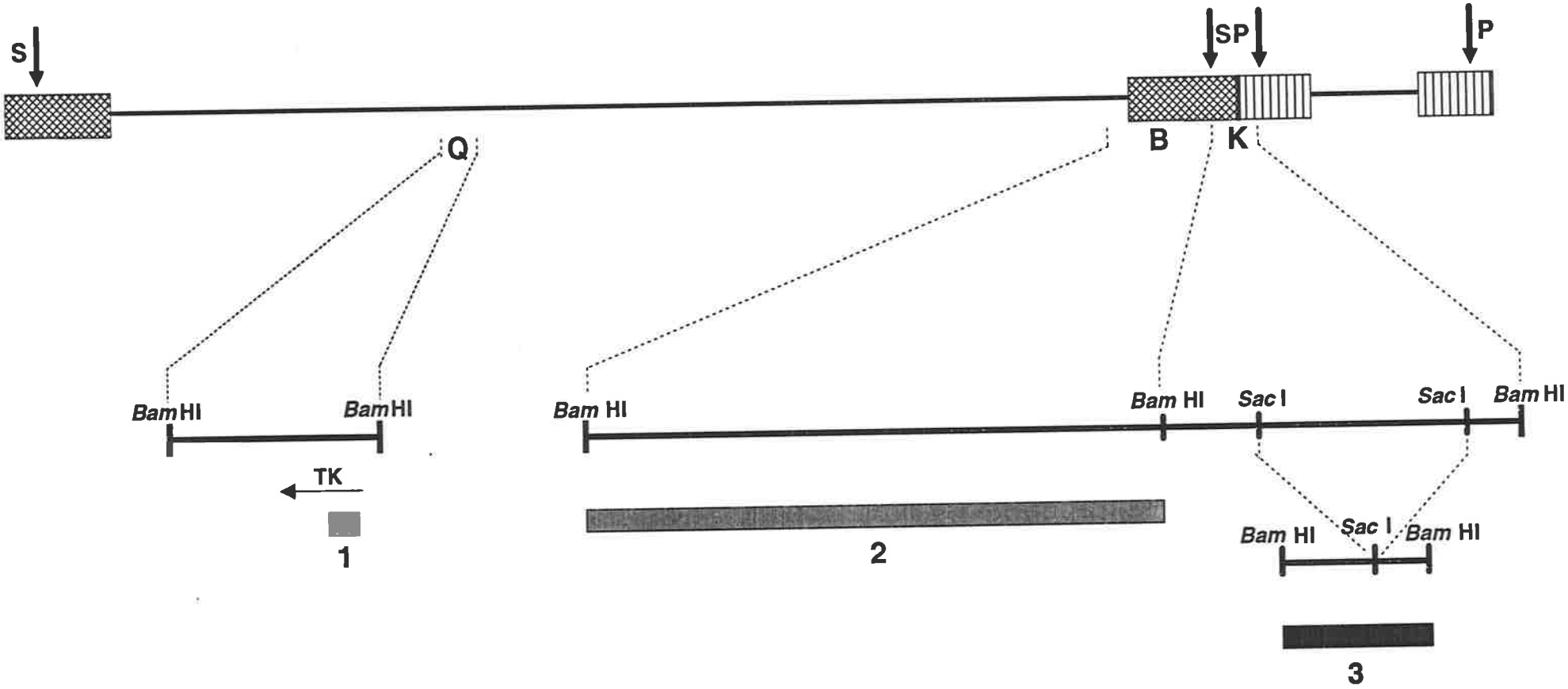
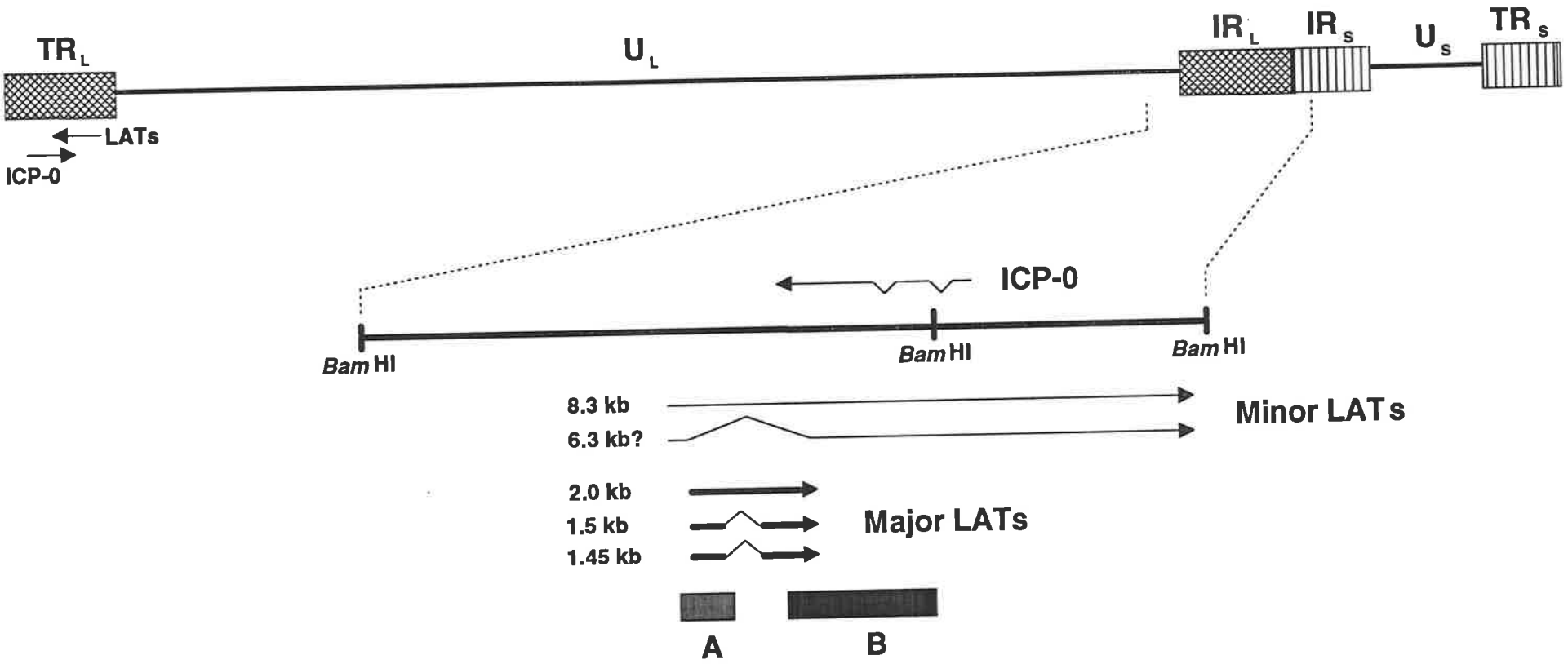

S
SP
P
B
K
Q
Bam HI
Bam HI
TK
1
Bam HI
Bam HI
Sac I
Sac I
Bam HI
2
Bam HI
Sac I
Bam HI
3

Figure 2.2

Map of the HSV-1 genome showing the positions of *Bam*HI-B sub-clones used as probes for the detection of LATs. Box A shows a 786 bp *Sph*I-*Sph*I fragment, which overlaps major (thick arrows) and minor LATs (thin arrows), used to construct pSLAT-4. Box B shows a 2.5 kb *Bam*HI-*Sal*I fragment, overlapping major and minor LAT sequences, used to construct pBS-0. (L: long segment; S: short segment; TR_L: long terminal repeat region; U_L: long unique region; IR_L: long internal repeat region; IR_S: short internal repeat region; U_S: short unique region; TR_S: short terminal repeat region).


TR L
U L
IR L
IR S
U S
TR S
LATs
ICP-0
ICP-0
Bam HI
Bam HI
Bam HI
8.3 kb
6.3 kb?
Minor LATs
2.0 kb
1.5 kb
1.45 kb
Major LATs
A
B

mRNA. pSLAT-4 comprises a 786 bp *Sph*I-*Sph*I fragment of HSV-1 strain 17 cloned into Bluescribe M13$^-$ (Figure 2.2). Transcripts from the T7 promoter of pSLAT-4 are complementary to major and minor LATs.

pSB-6, a gift from S Efstathiou, Cambridge University, UK, contains the 10.1 kb *Bam*HI-*Bam*HI B fragment of HSV-1 strain SC16 cloned into the *Bam*HI site of pUC-13 (Figure 2.1).

pBKSP1, a gift from A Puga, National Institutes of Health, USA, contains the 5.9 kb *Bam*HI-*Bam*HI K junction fragment of HSV-1 strain KOS from which an internal 3.5 kb *Sac*I-*Sac*I fragment has been deleted and inserted into the *Bam*HI site of pBR322 (Figure 2.1).

pAL41, a gift from S Alonso, Pasteur Institute, France, contains a 1150 bp *Pst*I-*Pst*I fragment isolated from a mouse lymphocyte cDNA library, complementary to β-actin mRNA, inserted into the *Pst*I site of pBR322.

2.1.4 Buffers and solutions (alphabetical listing)

Decalcification solution: 30 mM NaH_2PO_4, 70 mM Na_2HPO_4, 200 mM Na_2EDTA, pH 7.4 (Brain, 1966).

Denaturation solution: 0.5 M NaOH, 1.5 M NaCl.

Denhardts solution (1x): 20 μg/ml polyvinyl pyrrolidone (PVP), 20 μg/ml bovine serum albumin (BSA), 20 μg/ml ficoll 400.

Digoxigenin (DIG) buffer 1: 100 mM Tris-HCl pH 7.5, 150 mM NaCl.

DIG buffer 2: 1% (w/v) Blocking Reagent for nucleic acid hybridization (Boehringer Mannheim) dissolved in DIG buffer 1.

DIG buffer 3: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$.

Guanidine acetate buffer (GAB): 6 M guanidine HCl, 200 mM sodium acetate pH 5.5.

HDMEM: 20 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) buffered Dulbecco modified Eagle's medium (Gibco BRL), 2.4 μg/ml penicillin, 3.2 μg/ml gentamycin.

HDMEM-GM (Growth medium): HDMEM + 10% fetal calf serum (FCS).

HDMEM-MM (Maintenance medium): HDMEM + 1% FCS.

Hybridization solution for northern blot hybridization: 1 x Denhardts solution, 5 x SSC, 0.1% SDS, 50% deionized formamide, 100 μg/ml sheared and denatured salmon sperm DNA, 10% dextran sulphate, 2.5 x 10^6 cpm/ml denatured ^{32}P-labelled DNA probe.

Hybridization solution for *in situ* hybridization: 50% deionised formamide (Davis *et al.*, 1986), 1x SSC, 1x hybridization buffer, 0.5 mg/ml sheared denatured salmon sperm DNA, 0.5 mg/ml tRNA, 20 mM DTT, 1u/μl RNAsin (ribonuclease inhibitor, Promega), digoxigenin labelled riboprobe.

Hybridization solution for Southern blot hybridization: 3 x Denhardts solution, 4 x SSC, 0.5% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 10% dextran sulphate, 2.5 x 10^6 cpm/ml ^{32}P-labelled DNA probe.

Hybridization buffer (5x) for *in situ* hybridization: 5 x SSC, 500 mM Tris-HCl pH 7.6, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 0.1% ficoll, 0.1% PVP.

MOPS buffer (1x): 20 mM MOPS, Free acid 3-[N-Morpholino] propanesulphonic acid (Sigma), 5 mM sodium acetate, 1 mM EDTA pH 7.0.

Neutralization solution: 3.0 M NaCl, 0.5 M Tris-HCl, pH 7.5.

Nitroblue tetrazolium chloride (NBT) solution: 75 mg/ml NBT in 70% (v/v) dimethylformamide.

PBS (phosphate buffered saline): 140 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4 (Sambrook *et al.*, 1989).

PLP (Periodate-lysine-paraformaldehyde): 10 mM sodium periodate, 75 mM lysine, 2% paraformaldehyde, 37 mM phosphate buffer pH 7.4 (McLean and Nakane, 1974).

Prehybridization solution for northern blot hybridization: 5 x Denhardts solution, 5 x SSC, 0.1% SDS, 50% deionized formamide, 200 μg/ml sheared and denatured salmon sperm DNA, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4.

Prehybridization solution for Southern blot hybridization: 2 x Denhardts solution, 6 x SSC, 0.5% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4.

SSC (1x): 150 mM NaCl, 15 mM trisodium citrate, pH 7.0.

TAE (1x): 40 mM Tris(hydroxymethyl)Aminomethane (Tris), 20 mM glacial acetic acid, 1 mM EDTA pH 8.0.

TE (1x): (10:1); 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, (20:50); 20 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0.

Transcription Buffer (5x): 200 mM Tris-HCl pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl.

UREA-SDS buffer: 7 M urea, 350 mM NaCl, 50mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.2% SDS.

X-Phosphate solution: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (X-Phosphate), toluidinium salt in 100% dimethylformamide.

2.2 Methods

2.2.1 Quantification of infectious virus

Plaque assays were performed in duplicate by the suspension method of Russell (1962). Serial dilutions of virus samples or tissue homogenates, in 0.9 ml HDMEM-MM, were added to 3 x 10^6 Vero cells in 0.1 ml of HDMEM-MM and shaken for 1 hour at room temperature. Following incubation, 4 ml of HDMEM-GM supplemented with 1% carboxymethylcellulose (Sigma) was added to each sample. Samples were plated in 6 cm tissue culture dishes (Nunc, Denmark) and incubated at 37°C for 2.5 days in a humidified 5% CO_2 atmosphere. Cell monolayers were fixed with 10% formalin for 15 minutes, washed briefly in tap water, stained with 0.1% toluidine blue for 10 minutes before washing briefly in tap water. Plaques were counted using a low-power dissecting microscope.

2.2.2 Infection of mice

Prior to infection, the left flanks of C57BL10 mice were shaved under light anaesthesia and depiliated with Nair (Carter Wallace). For infection with HSV-1 strain SC16, a 10 μl drop of virus containing 3 x 10^4 plaque forming units (pfu) was applied to the posterior flank just to the left of the spleen tip, corresponding to the 8th thoracic dermatome (T8). Using a 27 gauge hypodermic needle, skin was scarified 20 times through the virus suspension, giving a total scarified area of approximately 4 mm^2 (Simmons and Nash 1984,

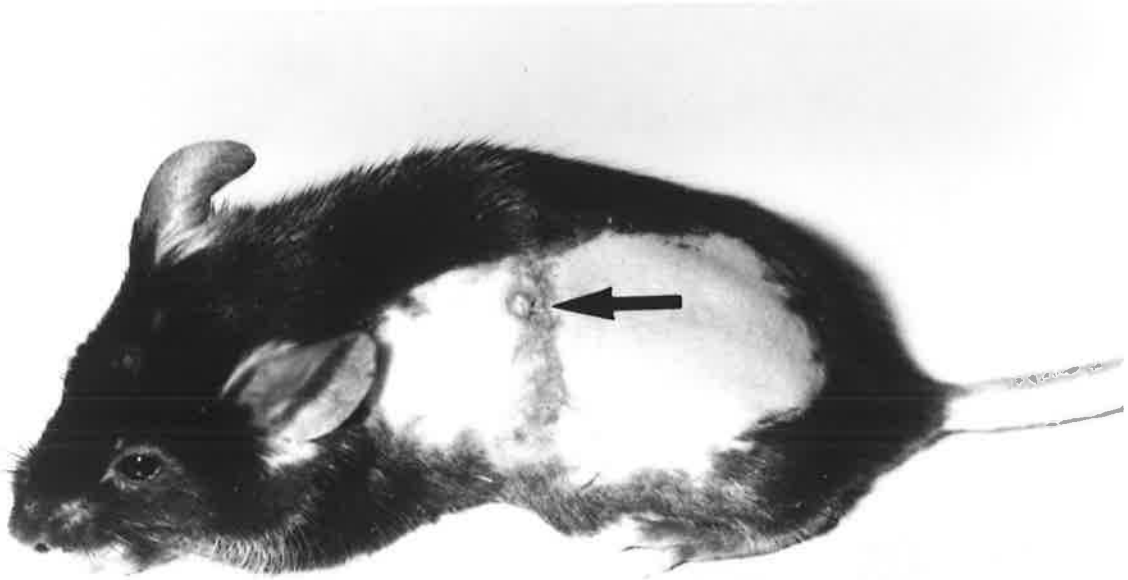
1985; Simmons Ph.D thesis, University of Cambridge, 1985; Simmons and La Vista, 1989; Speck and Simmons, 1991). Infected mice developed a characteristic band-like zosteriform lesion 5-6 days after virus inoculation (Figure 2.3). For infection with HSV-1 strain TKDM21, mice were infected at 3×10^7 pfu/10 μl drop and scarified over a wider area of flank skin. Mice infected with this virus displayed evidence of primary infection at the skin but no zosteriform lesion resulted.

2.2.3 Removal of infected tissue from mice

Mice were humanely killed either by CO_2 asphyxiation or intraperitoneal injection with 100 μl of sodium pentobarbitone (60 mg/ml). The viscera were removed through an anterior midline incision thus exposing the anterior surface of the spinal column. The thirteenth thoracic vertebra was identified by finding the thirteenth (lowest) rib, with which it articulates. Fine forceps were used to tease apart the vertebrae at the intervertebral discs in order to expose and remove consecutive dorsal root ganglia ipsilateral to the side of inoculation. Depending on the experiment, ganglia were either pooled or segmentally grouped according to anatomical location, eg. eighth thoracic ganglia (T8). Whole spinal sections were removed after exposing the anterior surface of the spinal column and dissecting away tissue attached to the vertebrae.

Figure 2.3

Characteristic band-like zosteriform lesion on the left flank of a C57BL10 mouse 5 days after infection with HSV-1 strain SC16 by scarification at one point. Arrow shows a primary lesion at the site of virus inoculation



2.2.4 Fixation of tissues

Dorsal root ganglia were fixed at room temperature for 2 hours in freshly prepared PLP. PLP was chosen because of its suitability for preserving nucleic acids (Moench *et al.,* 1985; Carmo-Fonseca *et al.*, 1991). Following fixation, ganglia were washed twice in 50% ethanol and rolled together into a ball.

Whole spines were fixed *in situ*. The thoracic cavity was opened and a small catheter inserted through the left ventricle into the aorta and clamped into place. Blood vessels were washed and dilated by introducing 150 mM $NaNO_2$ in PBS under pressure of 80-120 mm Hg. The right atrium was cut open to release circulated liquids. Animals were then perfused with PLP containing a trace of toluidine blue for 30 min. The success of the procedure was gauged by blue colour development in perfused tissue. Subsequently, spines were removed and to minimize distortion clamped firmly between rubber sponges, before fixation in PLP for a further 24 hours.

2.2.5 Tissue processing

Bones within whole spine sections were decalcified by immersion in decalcification solution for a period of 3 weeks. Tissue specimens were embedded with paraffin in a Shandon processing machine. To avoid shrinkage of neurons, the processing cycle included a gradual dehydration of tissues over several hours, using graded ethanol solutions.

2.2.6 Preparation of coverslips and slides and cutting of tissue sections

Glass slides and coverslips were immersed in acid wash solution for 16 hours, washed in 5 changes of double distilled water (DDW) and air dried. Coverslips were siliconized by dipping into 1% aqueous Prosil-28 (PCR Inc, Gainesville, Florida) for 5 seconds, rinsed twice in DDW, air-dried at 80°C for 2 hour and heat-sterilized. Slides were dipped in 2% aminopropyltriethoxysilane (APES)/ 98% ethanol for 20 seconds, rinsed three times in 100% ethanol, three times in DDW, airdried and stored for up to three months before use. APES coated slides were activated by immersion in 10% glutaraldehyde in PBS for 30 minutes (Maples, 1985) immediately before use. Ganglionic (5μm) and whole spinal coronal sections (5μm) were cut from paraffin blocks, floated on a 50°C water bath, collected onto activated APES coated slides, dried overnight at 37°C and stored until required.

2.2.7 Immunohistochemical detection of viral antigens

Viral antigens were detected in tissue sections using the peroxidase-anti-peroxidase method (Moriarty *et al.*, 1973; Sternberger, 1979; Boenisch, 1980). The primary antibody was rabbit antiserum to HSV infected cells (Dakopatts, Glostrup, Denmark), diluted 1/50. Binding of this antibody was detected using swine anti-rabbit immunoglobulin (diluted 1/25), followed by a 1/200 dilution of rabbit peroxidase-anti-peroxidase conjugate (all from Dakopatts). Sections were blocked for 30 minutes in 10% normal swine serum (NSS) prior to the addition of the primary antibody. All antibodies were diluted in 10% NSS. All

reactions were allowed to procede for 30 minutes at 37^{0}C with a 10 minute wash in 50 mM Tris buffer (pH 7.4) between steps. Bound antibody was detected with 3,3'-diaminobenzidine (0.5 mg/ml, containing 0.1% H_2O_2) for 4 minutes, then rinsed in water, counterstained with rapid haematoxylin, dehydrated through graded alcohol solutions and mounted in Depex (Ajax Chemicals, Sydney, Australia). Positive staining appeared as brown staining of ganglionic cells.

2.2.8 Extraction of ganglionic DNA

Ganglia removed from mice for DNA extraction were placed immediately into PBS on ice before being washed twice in PBS and transferred to a 1 ml tissue homogenizer. Ganglia were homogenized gently in 300 μl TE (20:50) pH 8.0 and sodium dodecyl sulphate (SDS) was then added, to a concentration of 0.5%. The lysate was treated with 100 μg/ml proteinase K (Boehringer Mannheim, Australia) for two hours at 37°C, and extracted three times with phenol-chloroform (1:1) and three times more with chloroform. A 1/10 volume of 3M sodium acetate (pH 5.5), followed by three volumes of cold 100% ethanol (-20°C), were added and precipitated DNA was spooled, airdried, and dissolved overnight at 4°C in cell culture grade sterile deionized water (Commonwealth Serum Laboratories, Australia, and herein referred to as CSL water). The amount and purity of total DNA from this procedure was assessed by spectrophotometry. Typical yields were 1μg of DNA per dorsal root ganglion. Samples were stored at -20°C until required.

2.2.9 Extraction of ganglionic RNA

To minimize the risk of RNA degradation, all glassware and materials were acid washed and/or heat sterilized and unless otherwise stated, the extraction procedure was carried out at 4°C.

Immediately after removal, dorsal root ganglia were snap frozen by placing into eppendorf tubes floating on liquid nitrogen. Ganglia were transferred to a 1 ml glass tissue homogenizer and homogenized vigorously in guanidine acetate buffer (GAB). A half volume of cold 100% ethanol was added and the homogenate incubated for 16 hours at -20°C. Samples were microcentrifuged at 12000 x g for 10 minutes, the supernatant discarded and the pellet resuspended by vigorous homogenization in approximately 300μl UREA-SDS buffer. Samples were extracted twice with phenol/chloroform (1:1) followed by the addition of a 1/10 volume of 3M sodium acetate (pH 5.5) and three volumes of cold 100% ethanol. RNA was precipitated for 16 hours at -20°C, pelleted at 12000 x g for 10 minutes, and resuspended in CSL water. Samples were then reprecipitated (1/10 volume sodium acetate, three volumes 100% ethanol, overnight at -20°C), repelleted (12000 x g), washed briefly in 95% ethanol, dried and resuspended in CSL water (50μl). The amount and purity of total RNA was assessed by spectrophotometry. Samples were stored at -70°C until required.

2.2.10 Preparation of probes for Southern and northern blot hybridization

2.2.10.1 Probe constuction

Inserts containing HSV-1 sequences were excised from pBAZ-1, pBKSP1, pSB-6 and pSLAT-4 by restriction endonuclease digestion with *Pst*I, *Bam*HI, *Bam*HI and *Sph*I respectively. The mouse β-actin insert was excised from pAL41 with *Pst*I. Digested DNA samples were electrophoresed on 1.0% TAE agarose gels and the HSV-1 DNA insert purified from host plasmid DNA by applying a Geneclean II kit (Bio 101 Inc.,California, USA) to agarose plugs containing the relevant DNA. Recovered insert DNA was quantified and checked for correct size by gel electrophoresis against known DNA size markers (*Eco*RI digested phage SPP1 DNA, Bresatec, Australia), and labelled with ^{32}P using an *in vitro* random primed DNA labelling procedure (Feinberg and Vogelstein, 1983). Reaction mixtures contained:

50 ng denatured DNA template

25 μM dATP (Boehringer Mannheim)

25 μM dGTP (Boehringer Mannheim)

25 μM dTTP (Boehringer Mannheim)

50 μCi ^{32}P-dCTP, 3000 Ci/mM (Bresatec, Australia)

1x random hexanucleotide primer reaction mixture (Boehringer Mannheim)

2 units Klenow enzyme labelling grade (Boehringer Mannheim)

CSL water to a final volume of 20 μl

The mixture was incubated at 37^{0}C for one hour before the reaction was stopped with 1 μl 500 mM EDTA pH 8.0. Efficiency of labelling was determined by measuring the incorporation of radioactive label by differential precipitation of DNA, using trichloroacetic acid (TCA). Labelled DNA was precipitated by adding 20 μl 3M sodium acetate pH 5.2, 20 μl 10 mg/ml sheared salmon sperm DNA, 40 μl CSL water and 450 μl cold 100% ethanol (-20^{0}C). The mixture was stored at -70^{0}C for four hours before centifugation at 12000 x g for 20 minutes. To remove unincorporated nucleotides the pellet was washed five times in 100% ethanol, dried briefly under vaccum and redissolved in 300 μl CSL water. Probes were utilized in hybridization reactions within two hours of construction.

2.2.10.2 Measurement of incorporation of radiolabelled nucleotides

Differential precipitation of nucleic acid by TCA was used to measure the efficiency of incorporation of radiolabel into DNA probes (Sambrook *et al.*, 1989). 1 μl samples of reaction mixture were spotted onto each of two 1 cm^2 discs of filter paper (Whatman S42) and air dried for 10 minutes. One filter was placed into liquid scintillation fluid (OptiPhase HiSafe 3, LKB Scintillation Products, UK) with no further treatment. This filter was used to measure the total radioactivity in the reaction (ie. incorporated and unincorporated radionucleotides). The other filter was washed in 10% TCA for 10 minutes to measure acid-precipitable radioactivity (ie. unincorporated radionucleotides). During this washing procedure, sequences greater than 50

nucleotides in length precipitate and are trapped in the filter paper, and unincorporated nucleotides elute from the filter (Sambrook *et al.*, 1989). Filters were dehydrated by successive washes in 100% ethanol, 50% ethanol/50% ether and 100% ether and placed into liquid scintillant. Radioactive counts were measured on a Beckman LS6800 scintillation counter. The proportion of incorporated radionucleotides was calculated by dividing the counts on the TCA treated filter by the counts on the unwashed filter. Typically, 75-90% of available radiolabel was incorporated into DNA sequences.

This procedure was also suitable to assess the incorporation of ^{32}P-rUTP radioactive tracer into RNA transcripts during synthesis of digoxigenin-labelled riboprobes.

2.2.10.3 Calculation of specific activity of radiolabelled DNA

The percentage incorporation of radionucleotides can be used to calculate probe specific activity. Probes were typically labelled to a specific activity of 10^9 dpm/μg as shown by the following example:

% incorporation of radionucleotides:	eg. 85%
Specific activity of ^{32}P-dCTP:	3000 Ci/mM
Number of μCi added:	50 μCi
Amount of input DNA:	50 ng

Because random primed DNA labelling leads to net DNA synthesis, the total amount of DNA at the end of the reaction must first be calculated.

(A) Total amount of DNA (ng) =

(number μCi added x 13.2 x % incorporation/specific activity ^{32}P-dCTP)

+ amount of input DNA

= (50 x 13.2 x 85/3000) + 50 = 68.7 ng

(B) Amount of radioactivity incorporated in the reaction (dpm) =

number μCi added x 2.2 x 10^4 x % incorporation

= 50 x 2.2 x 10^4 x 85 = 9.35×10^7 dpm

(C) Specific activity of labelled DNA (dpm/μg)=

(amount radioactivity incorporated/total amount DNA) x 1000

= 9.35 x 10^7/68.7 x 1000 = 1.4×10^9 dpm/μg

2.2.10.4 *Calculation of Tm_{50}*

For *in situ* hybridization experiments the temperature at which 50% of double stranded RNA hybrids will dissociate in liquid into single stranded molecules (Tm_{50}) is defined by the equation:

$$Tm_{50}(RNA/RNA) = 79.8 + 18.5\log[Na^+] - (0.35 \times \%formamide) + 58.4 \times (\%G+C) + 11.8 \times (\%G+C)^2$$

For Southern blot hybridization experiments the Tm_{50} for double stranded DNA hybrids is:

$$Tm_{50}(DNA/DNA) = 16.6\log[Na^+] + 0.41(\%G+C) + 81.5 - 0.75(\%formamide)$$

For northern blot hybridization experiments the Tm_{50} for double stranded DNA/RNA hybrids is defined as the average of the Tm_{50} values for RNA/RNA and DNA/DNA hybrids:

$$Tm_{50}(RNA/DNA) = [Tm_{50}(RNA/RNA) + Tm_{50}(DNA/DNA)]/2$$

(Meinkoth and Wahl, 1984; Bodkin and Knudson, 1985; Sambrook *et al.*, 1989)

2.2.11 Southern blot hybridization

2.2.11.1 Preparation of viral DNA 'copy per cell' reconstructions

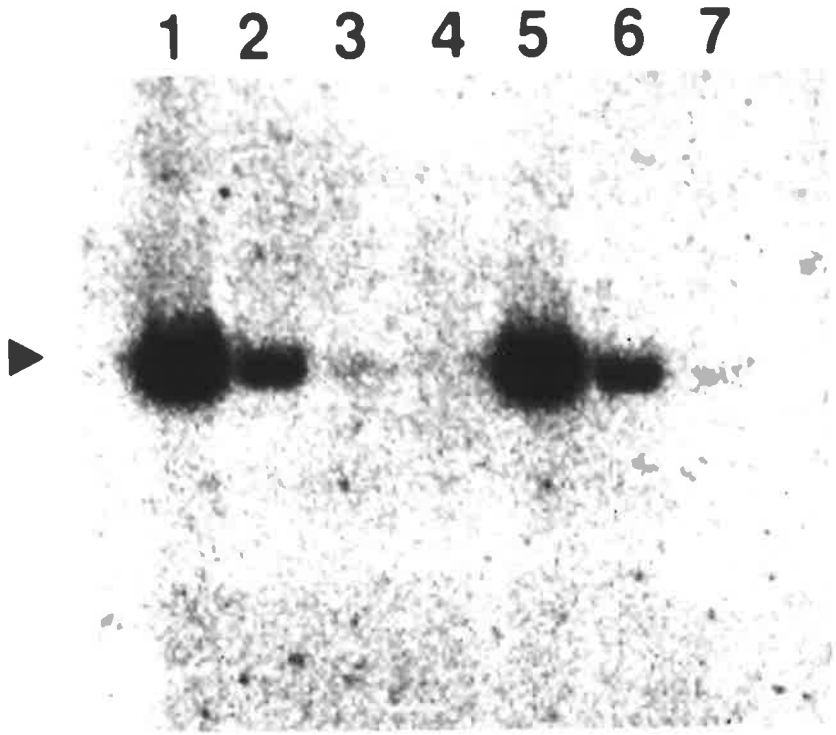
Viral DNA 'copy per cell' reconstructions were made to enable accurate assessment of the number of HSV-1 genome copy equivalents in ganglionic samples. Known amounts of HSV-1 virion DNA or insert DNA from pBAZ-1 were mixed with uninfected mouse DNA, to give the equivalent of 5, 0.5 or 0.05 viral DNA copies per mouse cell. Reconstructions made with virion DNA and plasmid insert DNA were shown to be comparable by Southern blot analysis with a probe derived from the pBAZ-1 HSV-1 insert (Figure 2.4).

2.2.11.2 Endonuclease digestion, electrophoresis and Southern blot transfer

DNA samples (10 μg) were restriction endonuclease digested with 40 units *Bam*HI (Amersham) for 2 hours at 37°C. All experiments included DNA extracted from uninfected mice, and DNA size markers were included, where applicable, to assist in determination of band size. A 1/10 volume of blue tracking dye was added and samples were loaded into a 0.8% TAE agarose gel containing 1 μg/ml ethidium bromide. DNA was electrophoresed at 20 volts for 16 hours, after an initial running-in period of 100 volts for 10 minutes. The gel was destained in DDW for 10 minutes, placed onto an ultraviolet transilluminator (UVP, San Gabriel, USA), photographed, and the DNA partially nicked by exposure to ultraviolet (UV) light at a wavelength of 302 nm for 2 minutes. The gel was then shaken gently for 35 minutes, first with

Figure 2.4

Detection of HSV DNA sequences in viral genome 'copy/cell' reconstructions using Southern blot hybridization and PhosphorImage analysis. Reconstructions were made either with pBAZ-1 DNA (lanes 1 to 3) or purified HSV-1 strain SC16 virion DNA (lanes 5 to 7). Samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose, and hybridized to a ^{32}P-labelled HSV TK probe derived from pBAZ-1. pBAZ-1 reconstructions were made by mixing 40 pg (lane 1), 4 pg (lane 2) and 0.4 pg (lane 3) of plasmid DNA with 10 μg uninfected mouse spleen DNA, corresponding to 5, 0.5 and 0.05 viral genome equivalent copies/cell, respectively. SC16 reconstructions were made by mixing 1.65 ng (lane 5), 0.165 ng (lane 6) and 0.0165 ng (lane 7) virion DNA with 10 μg uninfected spleen DNA, corresponding to 5, 0.5 and 0.05 genome copies/cell, respectively. Lane 4 contains uninfected mouse spleen DNA. The arrowhead marks the position of the 3.5 kb *Bam*HI-Q fragment of HSV-1 strain SC16.


1 2 3 4 5 6 7

denaturation solution and then neutralization solution, before DNA was transferred to nitrocellulose filters (Schleicher and Schuell Co, Germany) by the technique of Southern (1975). After transfer, filters were rinsed briefly in 2 x SSC, baked under vaccum at 80^0C and stored between sheets of Whatman 3MM chromatography paper (Whatman International Ltd, Maidstone, UK) until required.

2.2.11.3 Prehybridization and hybridization

Nitrocellulose filters were wetted in 6 x SSC and carefully placed into siliconized (Coatasil, Ajax Chemicals, Australia) 30 cm glass hybridization tubes (Robbins Scientific). Filters were prehybridized at 65^0C for 4 hours in 20 ml prehybridization solution (for DNA hybridization) in a rolling hybridization incubator, model 310 (Robbins Scientific). Hybridization was performed at 65^0C in 20 ml hybridization solution (for DNA hybridization) for 16-18 hours. Hybridization solution contained 5 x 10^7 cpm ^{32}P-labelled denatured DNA probe at a specific activity of approximately 10^9 dpm/μg, which corresponded to approximately 4 ng labelled DNA per ml hybridization solution.

2.2.11.4 Washing

All washes were performed using prewarmed solutions in a rolling hybridization oven. To remove unbound probe, filters were washed 4 times for 1 minute and once for 30 minutes in 2 x SSC, 0.1% SDS at 65^0C before being washed at high stringency for 2 x 45 minutes in 0.1 x SSC, 0.1% SDS at 65^0C.

Filters were wrapped without drying in plastic wrap (Vitafilm, Goodyear) and exposed to PhosphorImage storage screens for 1-2 days.

2.2.11.5 Detection of bound probe by phosphor imaging

Storage phosphor technology was utilized to detect and quantify specific hybridization signal. Phosphorimaging provided an alternative to conventional autoradiography by using a photostimulable storage phosphor screen instead of X-ray film. This system is more sensitive (15-fold higher for ^{32}P) and the dynamic range of accurate quantification is five orders of magnitude which is 200 times greater than X-ray film (Johnston *et al.*, 1990). Thus, PhosphorImage analysis combines high sensitivity with a significant reduction in autoradiographic exposure times. In addition, digital storage of images enables the degree of hybridization to be quantified accurately.

Exposed phosphor screens were analysed by a 400 series PhosphorImager (Molecular Dynamics, California, USA). Bands were visualized and, where applicable, quantified in relation to reconstructions, using ImageQuant software (version 3.0, Molecular Dynamics).

2.2.12 Northern blot hybridization

2.2.12.1 Electrophoresis and northern blot transfer

RNA samples (5 μg) were mixed with three volumes of a solution containing deionized formamide, formaldehyde and 10 x MOPs buffer at a ratio of 5:1.5:1, and heated to 70^{0}C for 5 minutes. A 1/10 volume of loading dye containing 50% glycerol, 1 x MOPS buffer and 0.25% bromophenol blue was added to each sample. Duplicates of each sample were loaded into a 1% agarose gel containing 1 x MOPS buffer and 2% filtered fresh formaldehyde, and electrophoresed in 1 x MOPS buffer at 70 volts for 4 hours. The gel was then cut in half; one set of duplicate samples was stained with ethidium bromide and photographed whilst the other was soaked in 20 x SSC and transferred to nitrocellulose (Schleicher and Schuell Co, Germany) by standard northern blotting (Sambrook *et al.*, 1989). After transfer, filters were baked for 2 hours at 80^{0}C, rinsed briefly in 2 x SSC (to remove excess salt crystals), air dried and stored between sheets of Whatman 3MM chromatography paper until required. Uninfected ganglionic RNA controls were included in all experiments.

2.2.12.2 Prehybridization and hybridization

Filters were wetted in 5 x SSC, placed into siliconized (Coatasil, Ajax Chemicals) hybridization tubes (Robbins Scientific) and prehybridized in 20 ml prehybridization solution (for RNA hybridization) for 4 hours at 42^{0}C in a rolling hybridization incubator (Robbins Scientific). Filters were hybridized for

16-18 hours at 42^{0}C in 20 ml hybridization solution (for RNA hybridization), containing 5 x 10^7 cpm ^{32}P-labelled denatured pSLAT-4 insert (specific activity of 10^9 dpm/μg, and approximately 4 ng labelled DNA per ml hybridization solution).

2.2.12.3 Washing and detection of bound probe

All washes were performed using prewarmed solutions in a rolling hybridization incubator (Robbins Scientific). Filters were initially washed 4 times for 1 minute and once for 30 minutes in 2 x SSC, 0.1% SDS at 70^{0}C. They were then washed at high stringency for 2x45 minutes in 0.1 x SSC, 0.1% SDS at 70^{0}C (Tm_{50}-16^{0}C). Filters were wrapped without drying in plastic wrap (Vitafilm, Goodyear), and exposed either to X-ray film (X-AR film, Kodak) at -70^{0}C, or storage phosphor screens at room temperature, for 1-3 days. X-ray film was developed using an Ilfospeed 2240 X-ray processor (Ilford). Phosphor screens were processed using a 400 series PhosphorImager (Molecular Dynamics), and the relative amounts of RNA in bands was determined using ImageQuant software (version 3.0, Molecular Dynamics).

2.2.12.4 Stripping nitrocellulose filters

To facilitate reprobing, filters were stripped of bound probe using the following method: A solution containing 0.05 x SSC and 10 mM EDTA pH 7.5 was heated to boiling prior to the addition of SDS to a final concentration of 0.1% (w/v). Filters were immersed this above solution for 15 minutes and the

procedure repeated with a fresh solution before being rinsed briefly in 0.01 x SSC at room temperature. Stripping was confirmed by exposing filters to phosphor screens prior to reprobing.

2.2.13 *In situ* hybridization

2.2.13.1 Choice of probes

Riboprobes (RNA probes) were utilized for the following reasons: (i) being single-stranded, the chance of self-annealling during the hybridization reaction is very low, which effectively increases probe concentration and enhances sensitivity (Angerer *et al.,* 1987) and (ii) when applied for the detection of RNA opposite sense transcripts can be used as a negative control for non-specific probe binding (Gowans *et al.,* 1989).

2.2.13.2 Choice of indicator molecule

Riboprobes were labelled with digoxigenin (DIG), a steroid hapten. The DIG system offers several significant advantages over conventional radioactive indicator molecules such as ^{35}S, ^{3}H, ^{32}P and ^{125}I which are commonly used in *in situ* hybridization (ISH). DIG is non-radioactive and therefore is safer than radioactive labels. Significantly, the same probe stock is stable for at least one year and can be used repeatedly, thus increasing the degree of reproducibility between experiments. Turnover time of experiments is greatly reduced because newly synthesized probes are not required for each ISH and the development time is typically measured in hours rather than days or weeks. In addition,

colour development can be monitored without stopping the reaction and signal is much more localized compared with emulsion autoradiography.

2.2.13.3 Preparation of digoxigenin-labelled riboprobes

Transcription vectors (either pBS-0 or pSLAT-4) were linearized by restriction endonuclease digestion such that the cloned fragment of interest was downstream of the appropriate promoter (ie. T3 or T7). Linearization was confirmed by electrophoresis on a 1% TAE agarose gel. The transcription reaction was carried out using a modification of the procedure described by Promega: Mixtures contained:

1 x Transcription buffer (Promega)

5 mM dithiothreitol (DTT) (Promega)

20 units RNAsin (ribonuclease inhibitor, Promega)

0.5 mM rATP (Promega)

0.5 mM rCTP (Promega)

0.5 mM rGTP (Promega)

0.085 μM ^{32}P-rUTP (Bresatec)

250 μM digoxigenin-11-rUTP (DIG-UTP; Boehringer Mannheim)

1 μg linearized template plasmid DNA

15 units T3 or T7 RNA polymerase (Promega)

CSL water to give a final reaction volume of 20 μl

The reaction mixture was incubated at 37°C for 1 hour. To digest away the DNA template, 1 unit of RNAse-free DNAse (Promega) was added and samples were incubated at 37°C for a further 15 minutes. The reaction was stopped with 1 μl 500 mM EDTA pH 8.0. The efficiency of transcription was determined by measuring the incorporation of the radioactive tracer by differential precipitation of RNA by trichloroacetic acid (TCA) precipitation (assuming that ^{32}P-rUTP and DIG-UTP were equally incorporated). To precipitate RNA transcripts, 20 μl 4 M LiCl and 20 μl 10 mg/ml sheared salmon sperm DNA were added, the mixture was brought to a final volume of 100 μl with CSL water and 250 μl of 100% ethanol (-20°C). The mixture was stored at -20°C overnight before centrifugation at 12000 x g for 20 minutes. The pellet was washed 4 times in 100% ethanol, once in 70% ethanol, dried under vaccum and redissolved in 100 μl 10 mM Tris-HCl pH 8.0 containing 1 mM DTT and 100 units RNAsin. Probes were stored at -20°C until required.

2.2.13.4 Preparation of tissue sections for ISH

The method used was a modification of the protocol described by Gowans *et al* (1989). Paraffin sections (5 μm) were dewaxed in two changes of xylene for 40 minutes and rehydrated gradually through graded ethanol/water mixtures over a period of 1 hour. Finally, slides were immersed in PBS. Sections were fixed in 0.1% glutaraldehyde in PBS for 30 minutes at 4°C, then washed twice for 5 minutes in PBS. Proteinase K digestion of tissue sections improves the access of probes but over-digestion results in poor morphology and loss of

target sequences (Angerer *et al.,* 1987; Gowans *et al.,* 1989). It was found that digestion with 100 μg/ml proteinase K (Boehringer Mannheim) in 20 mM Tris-HCl pH 7.5, 2 mM $CaCl_2$ at 37°C for 15 minutes, provided strong signal and good preservation of tissue morphology. Sections were washed twice for 5 minutes in PBS, refixed in 0.1% glutaraldehyde for 15 minutes at 4°C and treated with 0.25% triethanolamine pH 8.0 for 10 minutes at room temperature (Hayashi *et al.,* 1978). Following a further 2x 5 minute PBS washes, sections were gradually dehydrated in graded ethanols and air dried in preparation for the application of hybridization solution.

2.2.13.5 Nuclease digestions

Where indicated, tissues were treated with either 1 unit/μl DNAse 1 (Promega) and 6 mM $MgCl_2$, 40 mM Tris-HCl pH 7.5, for 1 hour at 37°C, or 500 μg/ml RNAse A (Pharmacia) in 2 x SSC for 1 hour at 37°C. Nuclease digestions were carried out after proteinase K digestion and tissue sections were thoroughly washed in PBS (5x5 minutes) before and after enzyme treatment.

2.2.13.6 Hybridization

Hybridization solution (20 μl) was applied to sections and covered with a 22 mm x 22 mm siliconized coverslip, taking care not to trap air bubbles. Coverslips were sealed with rubber cement (Super Vulkarn, Maruni Industries) and slides were incubated at Tm_{50} -25°C (Cox *et al.,* 1984; Gowans *et al.,* 1989) for 16 hours. All experiments included sections of uninfected ganglia.

2.2.13.7 *Washing*

Rubber cement was removed and coverslips gently removed by immersion of slides in 2 x SSC. To remove unbound probe, slides were washed in 2 litres of 2 x SSC for 1 hour and 2 litres of 0.1 x SSC for 1 hour with gentle shaking. High stringency washing was performed at Tm_{50} -12°C (ie. 75°C) in 30% deionized formamide in 0.1 x SSC, 10 mM Tris-HCl pH 7.5 for 30 minutes with gentle shaking. Formamide concentration was chosen to give washing temperatures compatible with good tissue preservation. Finally, slides were washed at room temperature in 0.1 x SSC, 10 mM Tris-HCl pH 7.5 for 15 minutes before development.

2.2.13.8 *Colour development*

Bound DIG-labelled probe was detected by colourimetric reaction, using a method described by Boehringer Mannheim. Slides were transferred to coplin jars and washed 5 minutes in DIG buffer 1 and blocked in DIG buffer 2 for 30 minutes with gentle shaking. Anti-DIG-alkaline phosphatase, F*ab* fragments (0.75 units/μl) was diluted 1/750 in DIG buffer 2 before being applied to individual tissue sections and incubated in a prewarmed humidified wet box at 37°C for 30 minutes. Slides were then placed back in coplin jars and washed in DIG buffer 1 (4 x 5 minutes) and DIG buffer 3 (1 x 5 minutes). Slides were immersed in freshly prepared colour substrate solution containing 135 μl NBT solution and 105 μl X-Phosphate solution in 30 ml DIG buffer 3. Colour development was examined intermittently and allowed to proceed for

approximately 2 hours before the reaction was stopped by washing slides in DDW. Positive probe hybridization for LATs appeared as strong blue/brown staining in neuronal nuclei.

3.DETECTION OF HSV NUCLEIC ACIDS DURING ACUTE AND LATENT GANGLIONIC INFECTION

To maximize survival of experimental animals, a genetically resistant strain of mice was selected for all experiments. C57BL10 mice are known to withstand at least 1000 times more virus than other inbred strains after inoculation of HSV-1 strain SC16 into flank skin (Simmons and La Vista, 1989). In order to achieve the aims of the project it was necessary to establish that HSV nucleic acids could be detected, by a variety of techniques, in the sensory ganglia of C57BL10 mice after cutaneous inoculation of virus.

3.1 Detection of HSV DNA in spinal ganglia of experimentally infected mice by Southern blot hybridization

The aim of the experiments described was to detect HSV DNA in sensory ganglia of acutely and latently infected C57BL10 mice. The mouse flank inoculation model utilized in these experiments has been partially characterized previously. Productive ganglionic infection, as determined by the presence of infectious virus, viral mRNA and the number of viral antigen positive neurons, peaks 5 days after inoculation of 3×10^4 pfu of HSV-1 strain SC16 into flank skin. Productive infection is then terminated rapidly over the next 2 days (Speck, 1991, PhD thesis, University of Adelaide; Speck and Simmons, 1991). Day 5 was chosen as an appropriate timepoint for detection and

characterization of acute-phase HSV DNA, and later times, *circa* day 24, were chosen for the study of latent viral DNA and RNA.

3.1.1 HSV DNA in acutely infected ganglia

Twelve adult C57BL10 mice were infected (day 0) with 3 x 10^4 pfu of HSV-1 strain SC16 by scarification of left flank skin in the T8 dermatome. On day 5, DNA was extracted from spinal ganglia (left, T8-T11) and digested with *Bam*HI. Samples (10 μg) were tested for the presence of HSV-1 DNA by Southern blot hybridization, using ^{32}P-labelled random primed DNA probes generated against three regions of the viral genome (Figure 2.1). Binding of probes was detected by PhosphorImage analysis after exposure to phosphor screens for 18 hours (Figure 3.1).

Probes generated from pBAZ-1, containing 875 bp of the HSV-1 thymidine kinase (TK) coding region, which is located within the U_L segment of the viral genome, hybridized to a 3.5 kb band corresponding to the expected size of the *Bam*HI-Q fragment of HSV-1 strain SC16 (Figure 3.1 A). Probe did not hybridize to DNA extracted from uninfected mice. It was concluded that, 5 days after inoculation of virus onto the flank skin of C57BL10 mice, viral DNA could be detected in sensory ganglia. This conclusion was confirmed using a second probe generated from pSB-6, which contains the *Bam*HI-B fragment of HSV-1. This probe detected two bands of approximately 10.1 kb and 8.9 kb, corresponding to the expected sizes of the viral *Bam*HI-B and

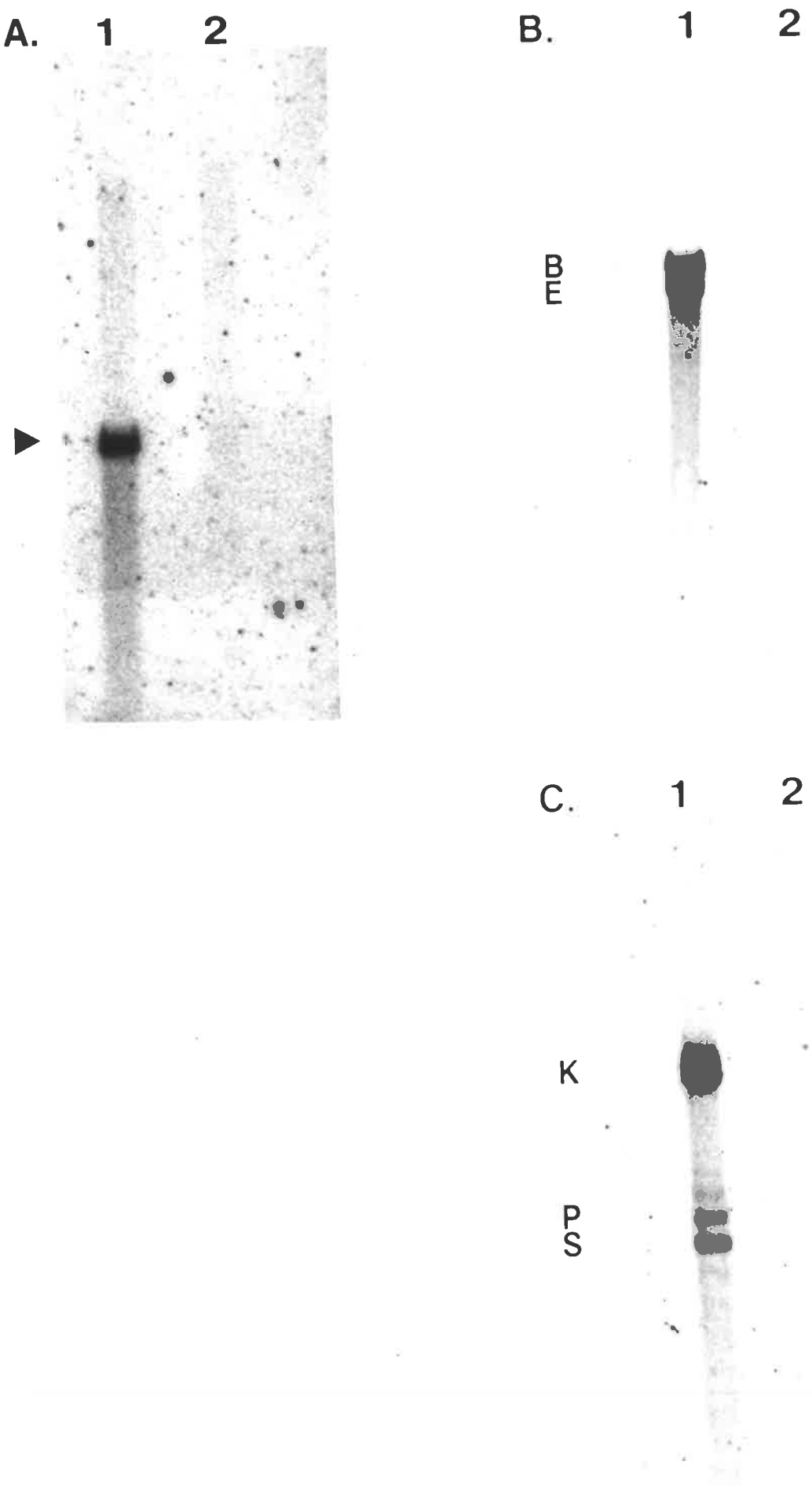
Figure 3.1

Detection of HSV DNA sequences in acutely infected spinal ganglia of C57BL10 mice 5 days after cutaneous inoculation with HSV-1 strain SC16. Ganglionic samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose by Southern blotting, and hybridized with ^{32}P-labelled probes derived from pBAZ-1, pSB-6 and pBKSP1 (A to C, repectively). Bound probes were detected by phosphor imaging.

(A) Phosphor image at 18 hours showing the detection of a 3.5 kb *Bam*HI-Q fragment of HSV-1 strain SC16 in acutely infected ganglia (lane 1, arrowed) probed with pBAZ-1. Hybridization to uninfected mouse spleen DNA was not observed (lane 2).

(B) Phosphor image at 18 hours showing HSV DNA sequences in acutely infected ganglia (lane 1) probed with pSB-6. The positions of the 10.1 kb *Bam*HI-B (B) and 8.9 kb *Bam*HI-E (E) fragments of HSV-1 strain SC16 are indicated. The probe did not hybridize to uninfected mouse spleen DNA (lane 2).

(C) Phosphor image at 18 hours showing the detection of junction (K; 5.9 kb) and terminal (P; 3.6 kb and S; 2.9 kb) fragments of HSV-1 in acutely infected ganglia (lane 1) probed with pBKSP1. The faint ladder of bands above the junction and terminal bands is caused by reiteration of the *a* sequence contained within the repeat regions of the viral genome. The probe did not hybridize to uninfected mouse spleen DNA (lane 2).


A.
1
2
B.
1
2
B
E
C.
1
2
K
P
S

*Bam*HI-E fragments, respectively (Figure 3.1 B). Detection of these two unimolar fragments was expected because the *Bam*HI-B probe contains part of both the unique and repeated sequences of the L segment. Hybridization to DNA extracted from uninfected mice was not observed.

A third probe, derived from pBKSP1 was used to provide limited structural information about viral DNA extracted from ganglia on day 5. pBKSP1 contains the *Bam*HI-K fragment from which an internal *Sac*I fragment has been deleted. The 2.5 kb fragment spans the L/S junction of the viral genome and is composed of a 1.7 kb repeated sequence from the L segment and a 0.8 kb repeated sequence from the S segment. The probe detected three major bands of approximately 5.9 kb, 3.6 kb and 2.9 kb, corresponding to the expected sizes of the *Bam*HI-K (junction), *Bam*HI-P (S, terminal) and *Bam*HI-S (L, terminal) fragments, respectively (Figure 3.1 C). The intensity of the *Bam*HI-P terminal band was slightly less than that of the *Bam*HI-S terminal band. This was not unexpected because the overlap of pBKSP1 with *Bam*HI-S is greater than with *Bam*HI-P (1.7 and 0.8 kb respectively). A ladder of weaker bands was also detected above the junction and terminal fragments. These bands were spaced at intervals of approximately 500 bp and are most likely due to reiteration of the *a* sequences, which are associated with the repeat segments of the viral genome. The relative intensities of the junction and two terminal fragments, determined by PhosphorImage analysis, was 3:1 (ie. for every pair of termini there were 3 junctions). Linear virion DNA has a junction:terminal

fragment ratio of 1:1. Based on the higher proportion of L/S junctions in acutely infected ganglia compared with virions, it was concluded that a proportion of viral genomes were not in a unit-length, linear configuration on day 5. Probe did not hybridize to DNA extracted from uninfected mice.

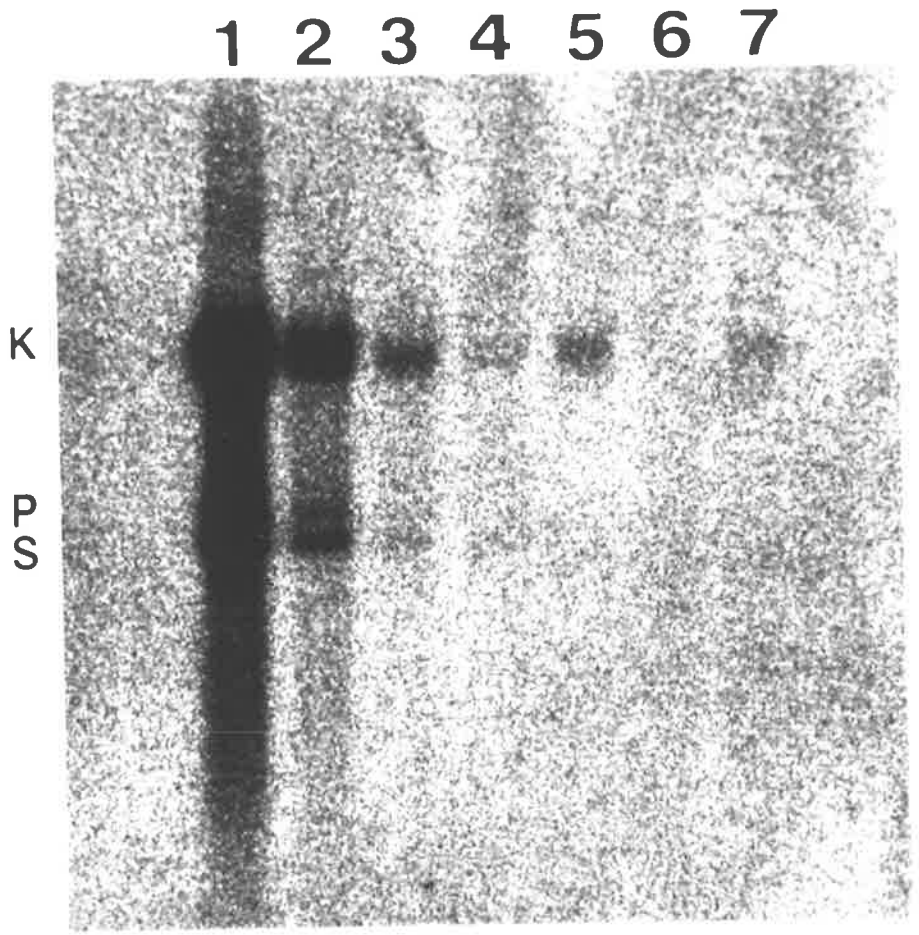
3.1.2 HSV DNA in latently infected ganglia

The ability to detect latent viral DNA was assessed by infecting C57BL10 mice with 3 x 10^4 pfu HSV-1 strain SC16 by scarification of left flanks in the T8 dermatome. DNA was extracted from spinal ganglia (left, T8-T11) 5 (12 mice), 24 (12 mice) and 192 (15 mice) days after infection. The day 5 DNA sample was serially diluted 1:5 in uninfected mouse cellular DNA and samples (10 μg), together with day 24 and day 192 material (10 μg each), were digested with *Bam*HI and analysed for the presence of viral DNA using a ^{32}P-labelled junction fragment probe derived from pBKSP1 (Figure 3.2).

Probe hybridized strongly to the day 5 (acutely infected) sample. Junction (K) and terminal fragments (P and S) of the HSV genome were detected at dilutions of 1:5, 1:25 and 1:125. At a dilution of 1:625 the junction fragment was visible but terminal fragments were at the limit of resolution. In contrast to day 5, no terminal fragments could be detected in DNA extracted 24 days after infection. The intensity of the junction fragment band on day 24 was similar to that of the 1:125 dilution sample of day 5 material. The presence of detectable terminal fragments in the 1:125 dilution of day 5 material, led to the

Figure 3.2

Detection of HSV DNA sequences by Southern blotting and PhosphorImage analysis (20 hour exposure) in spinal ganglia from C57BL10 mice 5 days (lanes 1 to 4), 24 days (lane 5) and 192 days (lane 7) after cutaneous inoculation with HSV-1 strain SC16. Lanes 1 to 4 contain 1:5, 1:25, 1:125 and 1:625 dilutions, respectively, of day 5 ganglionic DNA mixed with uninfected mouse spleen DNA. Samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose and hybridized with a ^{32}P-labelled HSV junction probe derived from pBKSP1. Note the presence of junction (K; 5.9 kb) and terminal (P; 3.6 kb and S; 2.9 kb) fragments in the day 5 sample, but the presence of only the K fragment in day 24 and day 192 samples. Lane 6 contains spleen DNA from uninfected mice.


1 2 3 4 5 6 7
K
P
S

conclusion that viral DNA 24 days after infection, is deficient in free genomic termini. The amount of viral DNA present on day 24 was approximately 125-fold less than the amount detected on day 5, indicating significant clearance of virus from sensory ganglia during this period.

Viral DNA detected in ganglia 192 days after infection resembled the day 24 material ie. the junction fragment but not terminal fragments were detectable. There was a small decrease in junction fragment band intensity at day 192 compared with day 24, but the decrease in latent viral DNA over this time was at most 2-fold. Probe did not hybridize to DNA from uninfected mice.

It was concluded that virus specific DNA sequences can be detected in sensory ganglia 5, 24 and 192 days after infection with a replication competent strain of HSV. Viral DNA detected on day 24 and day 192 was quantitatively and qualitatively different to that detected on day 5. Between days 24 and 192, the viral genome was maintained in a stable form that was deficient in free genomic termini, consistent with the establishment of latent infection.

3.2 Detection of HSV LATs in latently infected spinal ganglia by northern blotting and *in situ* hybridization

3.2.1 Detection of LATs by northern blot hybridization

Mice were infected (day 0) with HSV-1 strain SC16 (3 x 10^4 pfu) by scarification of the left flank. On day 25 spinal ganglia (left, T6-L1) were removed, total RNA was extracted and samples (5 μg) were analysed by northern blot hybridization using a ^{32}P-labelled DNA probe generated from pSLAT-4, which contains sequences spanning both major and minor LATs (Figure 3.3).

The probe hybridized to a band corresponding in size to the predominant major LAT species of approximately 2.0 kb; no other bands were detected. There was no detectable hybridization between the pSLAT-4 probe and RNA extracted from uninfected mice. It was concluded that the pSLAT-4 derived DNA probe specifically detected a 2.0 kb major LAT RNA species in ganglia of C57BL10 mice latently infected with HSV-1 strain SC16.

3.2.2 Detection of LAT$^+$ neurons by *in situ* hybridization

A high resolution, non-isotopic *in situ* hybridization (ISH) protocol was applied for the detection of LAT$^+$ neurons in latently infected spinal ganglia. C57BL10 mice were infected (day 0) with HSV-1 strain SC16 (3 x 10^4 pfu) by scarification of the left flank. On day 25, spinal ganglia (left, T8-L1) from 10

Figure 3.3

Autoradiograph (48 exposure) showing detection of LATs by northern blotting in latently infected spinal ganglia of C57BL10 mice 25 days after flank inoculation with HSV-1 strain SC16 (lane 2). RNA (5 μg) from pooled spinal ganglia (T6 to L1) was electrophoresed, transferred to nitrocellulose and hybridized to a ^{32}P-labelled LAT probe derived from pSLAT-4. The 2.0 kb major LAT species is arrowed. Lane 1 contains RNA (5 μg) extracted from spinal ganglia of uninfected mice (lane 1). Positions of the 1.9 kb 18s and 4.8 kb 28s ribosomal RNAs are indicated.

1 2

28s

18s

animals were studied by ISH using digoxigenin (DIG) labelled riboprobes derived from the T7 promoter of pBS-0 and pSLAT-4. Positive hybridization appeared as dark blue/brown staining in the nuclei of neurons (Figure 3.4). The number of positive neurons and the intensity of staining with labelled transcripts generated from the T7 promoter of either pBS-0 or pSLAT-4 (both of which are antisense to major and minor LATs) were comparable. The specificity of hybridization was assessed as follows:

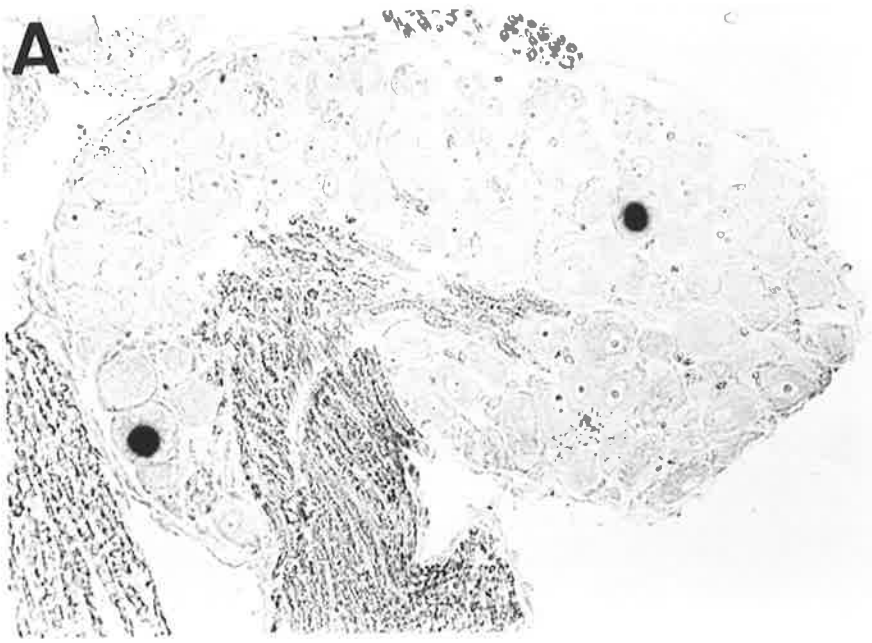
(a) Riboprobes generated from the T7 promoter of pBS-0 and pSLAT-4 hybridized strongly to ganglionic sections from latently infected mice (Figure 3.5 A) but did not hybridize to sections from uninfected animals. It was concluded that T7 derived riboprobes from pBS-0 and pSLAT-4 hybridized specifically to HSV nucleic acid sequences.

(b) Specificity of probes for HSV RNA rather than DNA was established in two ways:

(i) Prior to the addition of probes, sections were treated either with RNAse or DNAse. Pretreatment with RNAse resulted in complete loss of positive hybridization staining (Figure 3.5 B), whereas digestion of sections with DNAse did not significantly alter intensity of positive staining (Figure 3.5 C).

Figure 3.4

Detection of LATs by non-isotopic *in situ* hybridization in ganglia of C57BL10 mice 25 days after infection with HSV-1 strain SC16. Ganglionic sections were hybridized to digoxigenin-labelled riboprobes generated from the T7 promoter of pBS-0 (A; magnification x 96) and pSLAT-4 (B; magnification x 750). Bound probes were detected using phosphatase-conjugated anti-digoxigenin antibodies. Staining of LATs (black areas) was confined to neuronal nuclei and signal intensity was similar for both probes.


A

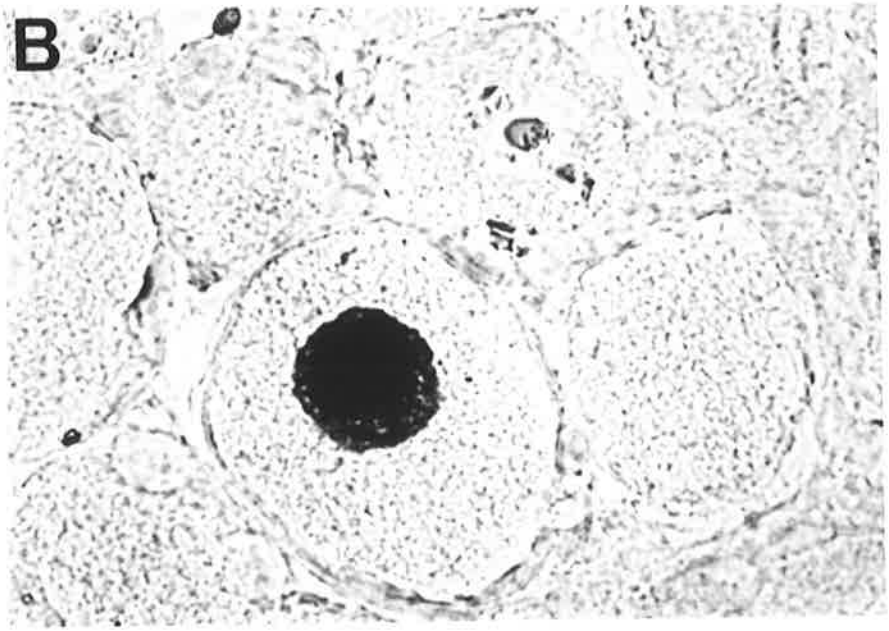
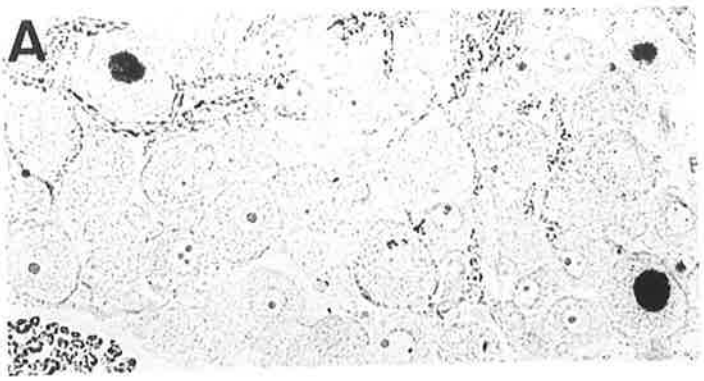
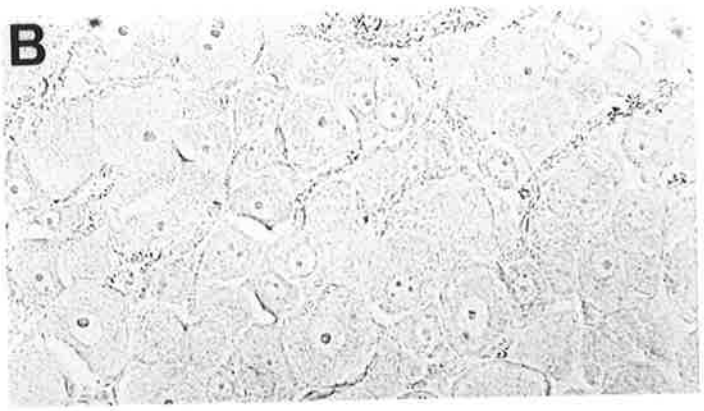
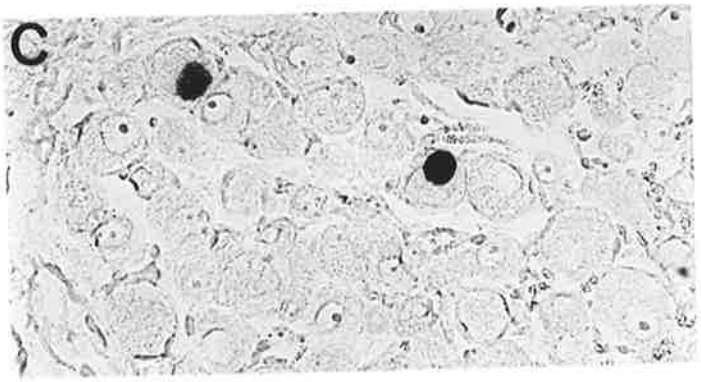

B

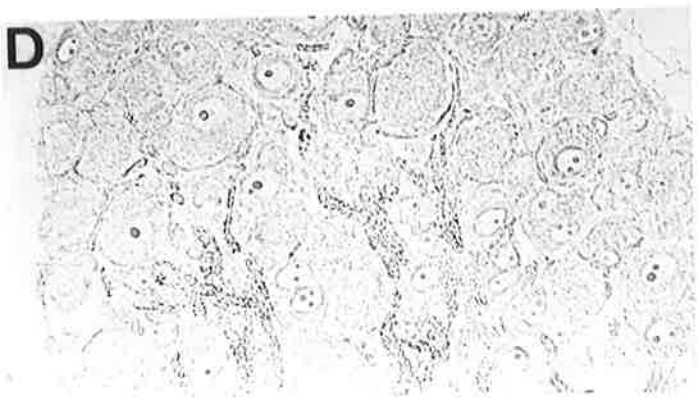
Figure 3.5

Validation of *in situ* hybridization for the detection of LATs in HSV infected ganglia. Photomicrographs (magnification x 300) of ganglionic sections of thoracic ganglia taken from C57BL10 mice 25 days after infection. Sections were untreated (A), digested with RNAse (B), or digested with DNAse (C) before hybridization to a DIG-labelled LAT riboprobe generated from the T7 promoter of pSLAT-4. Sections were also hybridized to a probe generated from the T3 promoter of pSLAT-4, which produces transcripts that are 'sense' to LATs (D). Note that LAT specific staining (black areas) was detected only in untreated and DNAse treated sections, probed with T7, pSLAT-4.


A


B


C


D

(ii) Single-stranded riboprobes generated from the T3 promoter of pBS-0 and pSLAT-4 are the same sense as LATs. In contrast to T7 transcripts these probes did not hybridize to sections of the same latently infected tissue (Figure 3.5 D).

It was therefore concluded that both pBS-0 and pSLAT-4 T7 transcripts specifically detected HSV RNA molecules *in situ* in latently infected ganglia of C57BL10 mice.

3.3 Discussion

3.3.1 HSV DNA

During the acute infection of C57BL10 mice with HSV-1 strain SC16, viral DNA sequences of predicted sizes were readily detected in thoracic dorsal root ganglia after inoculation of flank skin. At a timepoint previously shown to represent the peak of acute infection (day 5) the ratio between junction and terminal fragments of the viral genome was 3:1. The increased representation of junction fragments in acutely infected ganglia is analagous to the structure of replicating DNA in cultured cells and most likely represents the production of concatamers during rolling circle replication (Jacob *et al.*, 1979; Roizman, 1979a; Jongeneel and Bachenheimer, 1981; Poffenberger and Roizman, 1985). On days 24 and 192 genomic termini could not be detected in the HSV DNA recovered from ganglia. Absence of terminal fragments has been described previously in brainstems and trigeminal ganglia of BALB/c mice latently

infected with HSV-1 strain F (Rock and Fraser, 1983, 1985). Efstathiou *et al* (1986) confirmed the presence of 'endless' viral DNA in brainstems, spinal cord and cervical ganglia of latently infected BALB/c mice, and trigeminal ganglia of humans. This structurally distinct form of viral DNA may represent a covalently closed circular molecule or a concatemer comprised of a series of viral genomes linked head-to-tail, sequestered as episomes. Alternatively, the latent genome may exist as a unit-length or concatemeric molecule integrated into the host cellular genome. Novel bands corresponding to the virus-host junction would be expected if integration occurred via the repeat sequences at a limited number of specific sites in cellular DNA. Bands of this nature were not observed either on day 24 or day 192. However, by enriching for viral DNA using reverse-phase chromatography, Puga *et al* (1984) detected novel HSV fragments from the terminal repeats of the genome in latently infected mouse trigeminal ganglia. It is possible that enrichment resulted in an increase in sensitivity that enabled detection of a small number of virus-cell integrations, but as pointed out by Efstathiou *et al* (1986), the enrichment process may have also enriched cross-hybridizing cellular DNA sequences. Mellerick and Fraser (1987) used buoyant density centrifugation to demonstrate that the bulk of latent viral DNA sequences are sequestered in an extrachromosomal state. However, it remains possible that latent DNA contains a small proportion of integrated viral genomes, the remainder being sequestered in a circular or concatameric episomal state, resembling Epstein-Barr virus latency (Anvret *et al*., 1984).

3.3.2 Latency associated transcripts

By northern blot hybridization, a 2.0 kb major LAT species was readily detected in ganglia of latently infected mice, but previously reported 1.45 kb and 1.5 kb major LATs were not detected. There are two potential explanations for this observation. First, 1.45 kb and 1.5 kb LATs may have been present at levels too low to be detected by the northern hybridization procedure. For example, Steiner *et al* (1988) reported that in human trigeminal ganglia, a 1.5 kb major LAT species was present in low abundance relative to the 2.0 kb species. Second, it is possible that only a single LAT species of 2.0 kb is synthesized in C57BL10 mice latently infected with HSV-1 strain SC16. This is a likely possibility because the number of major LATs is known to be influenced by virus strain and host species. For instance, in Swiss-Webster mice latently infected with HSV strain KOS(M), 2 major LATs of 2.0 kb and 1.5 kb have been described (Wagner *et al.*, 1988a). All 3 variants (2.0 kb, 1.5 kb and 1.45 kb) have only been described in BALB/c mice latently infected with HSV-1 strain F (Spivak and Fraser, 1987, 1988b).

Neither an 8.3 kb primary transcript nor its putative spliced products (approximately 6.5-7.0 kb) were detected in the experiments described here. Detection of such transcripts in latently infected tissue by northern blot hybridization has been problematic and their existence remains to be proved with certainty (Zwaagstra *et al.*, 1989; Devi-Rao *et al.*, 1991). At this time, the best evidence in favour of the existence of a primary transcript, from which

major LATs might be excised by splicing, is detection of a low abundance 8.3 kb poly A^+ RNA species in acutely infected cells in culture (Dobson *et al.*, 1989; Mitchell *et al.*, 1990b; Zwaagstra *et al.*, 1990; Devi-Rao *et al.*, 1991).

3.3.3 LAT^+ neurons

By *in situ* hybridization, LATs were readily visualized in neurons of latently infected mice. As in previous studies, which used radioactive rather than non-isotopic probes, LATs were detected predominantly in neuronal nuclei. For the present study, non-isotopic detection methods were considered to offer several advantages over radiolabelled probes including (i) long shelf-life of digoxigenin-labelled probe preparations, (ii) ease and speed of development, (iii) lower cost, (iv) improved target localization ability, (v) increased safety and (vi) better signal to noise ratio.

4. RELATIONSHIP BETWEEN VIRAL DNA AND TRANSCRIPTIONAL ACTIVITY DURING LATENT INFECTION WITH A VIRULENT STRAIN OF HSV-1

The point at which molecular pathways leading to productive and latent infection diverge, and the related issue of whether viral DNA is amplified during establishment of latency, have been objects of speculation for many years. Typically, approximately 1% of ganglionic neurons appear to be latently infected, judged either by counting infectious centres after co-cultivating dissociated ganglia with susceptible cells (Walz *et al*, 1976; Nicholls & Blyth, 1989) or by enumerating LAT^+ cells by *in situ* hybridization (ISH) (eg. Tenser *et al*, 1989). On this basis, latently infected neurons have been calculated to each contain many copies of the viral genome (Efstathiou *et al*, 1986), suggesting that HSV DNA is amplified during or after establishment of latency. However, amplification of viral DNA is apparently not essential for establishment of latency because replication defective mutant virus can persist in the peripheral nervous system (PNS) of experimentally infected animals (Clements & Stow, 1989; Coen *et al*, 1989; Leib *et al*, 1989; Leist *et al*, 1989; Dobson *et al*, 1990; Kosz-Vnenchak, 1990; Steiner *et al*, 1990) and under these conditions the amount of HSV DNA recovered from latently infected ganglia is reduced (Efstathiou *et al*, 1989; Tenser *et al*, 1989; Friedrich *et al*, 1990; Katz *et al*, 1990; Valyi-Nagy *et al*, 1991). Therefore, it is likely that either the number of cells harbouring viral genomes has been underestimated or HSV

DNA is not distributed evenly amongst infected cells during latency.

In this and the subsequent chapter, these issues are investigated using an experimental system that makes novel use of segmental sensory innervation of flank skin. In mice, the thoracic and lumbar regions of the PNS are divided into 13 (T1-T13) and 5 (L1-L5) structural segments respectively, each innervating corresponding segments of skin (dermatomes). The distribution of acute and latent infection in the PNS can be mapped with precision after introduction of HSV-1 into the flank skin of C57BL10 mice (Speck & Simmons, 1991). Productive infection, judged by virus recovery and the presence of viral antigens and mRNAs, was restricted to ganglia innervating the site of inoculation, in contrast to latent infection, assessed by the presence of LAT^+ neurons and reactivation *in vitro*, which was much more widespread. Using this system the number of HSV genomes recovered from mouse spinal ganglia latently infected with HSV-1 strain SC16, was compared with the proportion of neurons containing LATs and the relative amounts of LATs in corresponding spinal segments.

4.1 Determination of HSV genome copy number per LAT$^+$ neuron in spinal ganglia T8-L1

C57BL10 mice were infected (day 0) with 3 x 10^4 pfu HSV-1 strain SC16 by scarification of the left flank in the region of the T8 dermatome. Ganglia spanning T8 to L1 were removed from the left side of latently infected mice on day 23, pooled with respect to vertebral level, and tested for the presence of either HSV DNA by Southern blot hybridization (62 animals) or LAT$^+$ neurons by *in situ* hybridization (25 animals).

4.1.1 Quantification of latent HSV DNA by Southern blot hybridization

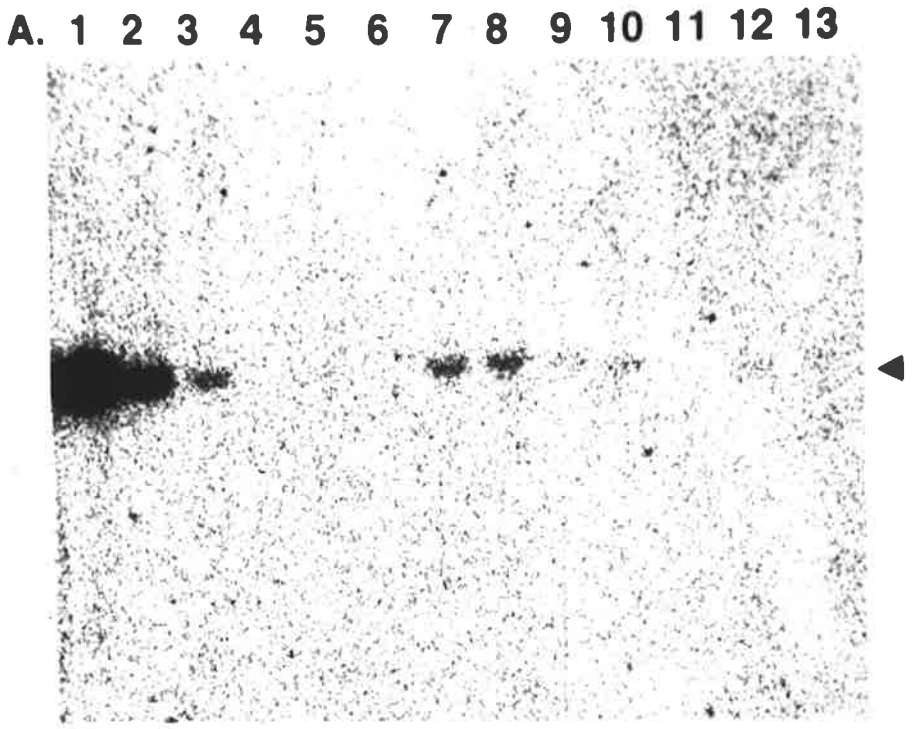
Segmentally pooled ganglionic DNA samples (10 μg) were digested with *Bam*HI and analysed by Southern blot hybridization using a ^{32}P-labelled DNA probe (pBAZ-1) from the thymidine kinase (TK) region of HSV-1. Filters were washed at a stringency of Tm_{50}-15^{0}C and exposed to phosphor screens for 17 hours. In latently infected ganglionic samples the probe hybridized to a single 3.5 kb band corresponding to the *Bam*HI-Q fragment of HSV-1 strain SC16, which contains the TK gene (Figure 4.1). The probe did not hybridize to DNA extracted from uninfected mice. Images were stored digitally and bands accurately quantified in relation to viral genome equivalent 'copy per cell' reconstructions (Table 4.1). Virus sequences were readily detected in ganglionic samples from T8 and T9, and were less abundant at T10 and T11 (Figure 4.1 A). A further analysis of samples between T10 and L1 showed,

Figure 4.1

Detection of HSV DNA sequences in latently infected spinal ganglia of C57BL10 23 days after flank inoculation with HSV-1 strain SC16. Samples (10 μg) were digested with *Bam*HI, Southern blotted and probed with a ^{32}P-labelled probe from pBAZ-1.

(A) Phosphor image at 17 hours showing distribution of viral DNA in spinal segments T8 to L1 (lanes 7 to 13), reconstuctions for quantification purposes using 5, 0.5 and 0.05 HSV genome equivalents/cell (lanes 1 to 3, respectively) and uninfected mouse spleen DNA (lane 5). Arrowhead marks the position of the 3.5 kb *Bam*HI-Q fragment of HSV-1.

(B) Phosphor image at 48 hours comparing reconstructions using 5, 0.5, and 0.05 genome equivalents/cell (lanes 1 to 3, respectively) and uninfected mouse spleen DNA (lane 5) with material from T10 to L1 only (lanes 7 to 11). Arrowhead marks the position of the 3.5 kb *Bam*HI-Q fragment.


A. 1 2 3 4 5 6 7 8 9 10 11 12 13

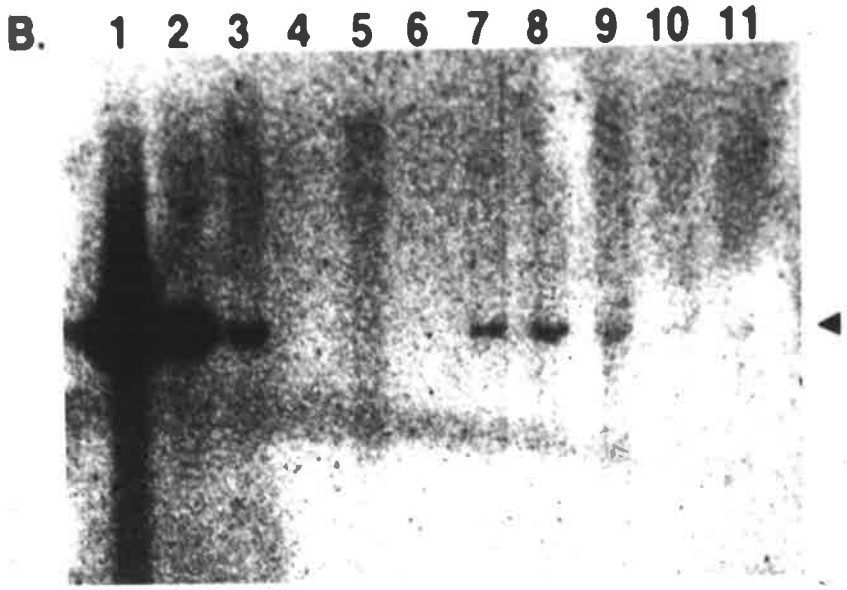

B. 1 2 3 4 5 6 7 8 9 10 11

TABLE 4.1 RELATIONSHIP BETWEEN HSV DNA AND LAT$^+$ NEURONS IN SPINAL SEGMENTS T8-L1

Spinal segment	HSV genome equivalents/cell	LAT$^+$ profiles/ section ± SEM (%)[a]	HSV genome equivalents/LAT$^+$ neuron[b]
T8	0.056	0.25 ± 0.04 (0.20)	280
T9	0.048	0.3 ± 0.06 (0.23)	209
T10	0.016	1.19 ± 0.21 (0.93)	17
T11	0.014	0.75 ± 0.11 (0.58)	24
T12	0.006	0.44 ± 0.05 (0.34)	18
T13	~0.001	0.17 ± 0.04 (0.13)	8
L1	~0.001	0.08 ± 0.02 (0.06)	17

a The percentage of LAT$^+$ neuronal profiles/ganglionic section were calculated using an estimate of total neuronal profiles/ganglionic section of 128.4 ± 3.3 (± SEM). This estimate was derived from pooled ganglia from C57BL10 mice (D Tscharke and A Simmons - unpublished results).

b Number of genome equivalents per LAT$^+$ neuron calculated by assuming that 10% of ganglionic cells are neurons (Walz *et al*, 1976).

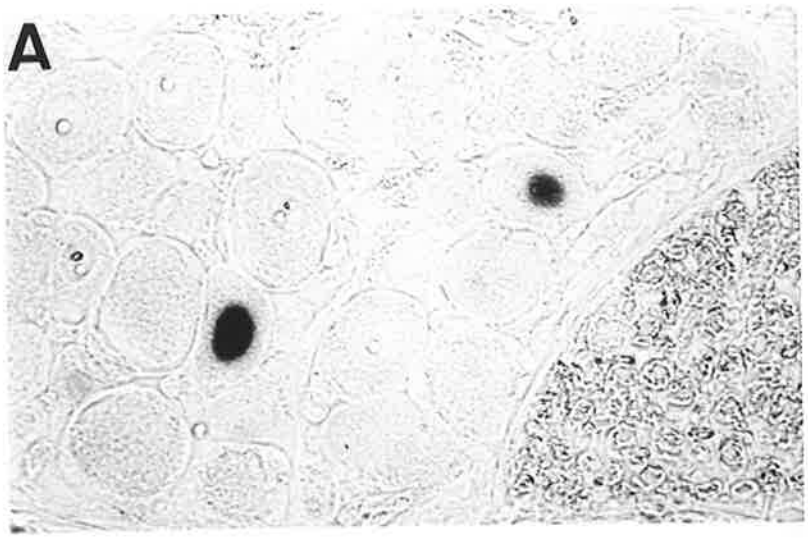
after 48 hour exposure to a phosphor screen, a small but quantifiable amount of DNA at T12 (Figure 4.1 B) in addition to the DNA previously identified at T10 and T11. Extremely faint signals (approximately 0.001 copies/cell) were detected in samples from T13 and L1, but these could not be precisely quantified. It was concluded that latent viral DNA sequences were distributed unevenly among ganglia from different spinal segments, and were most abundant in ganglia that innervated the inoculation site.

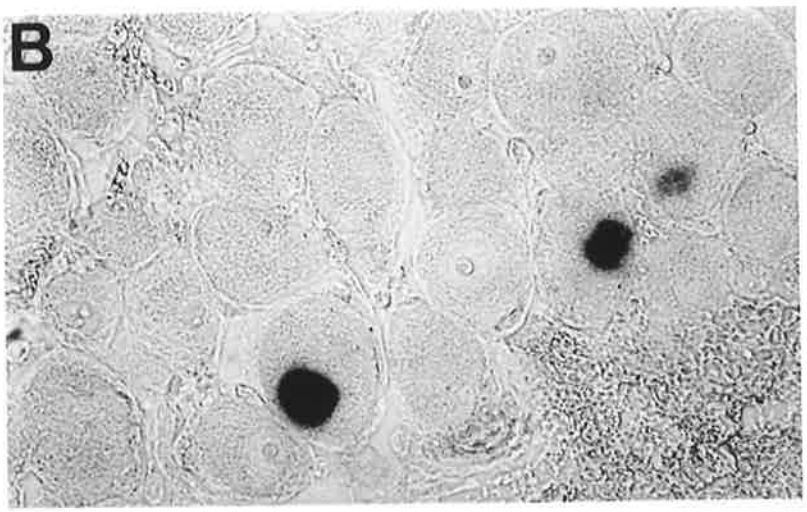
4.1.2 Enumeration of LAT$^+$ neurons by *in situ* hybridization

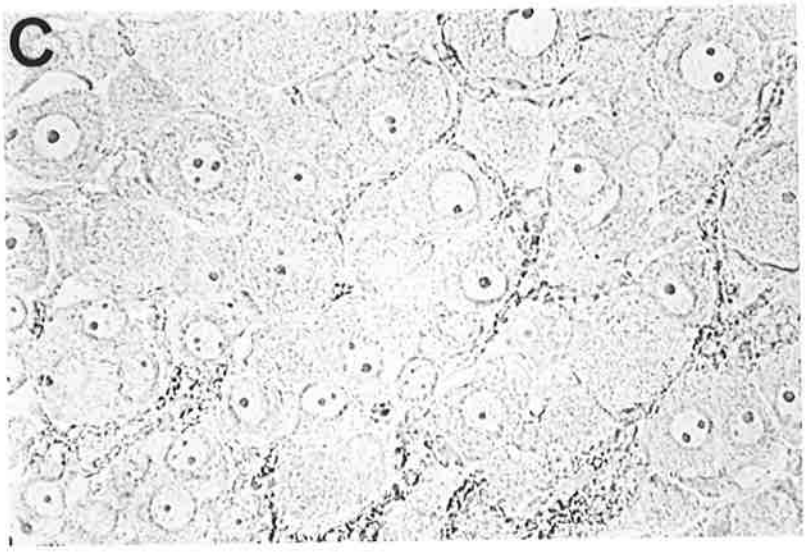
To assess accurately the relative number of LAT$^+$ primary sensory neurons at each spinal segment, an exhaustive examination was undertaken by *in situ* hybridization. Segmentally pooled ganglia were fixed, embedded in paraffin and 5 μm sections hybridized with a DIG-labelled riboprobe generated from the T7 promoter of pBS-0, which is complementary to major and minor LATs. Positive hybridization appeared as blue/brown staining in neuronal nuclei of latently infected ganglia (Figure 4.2). Probe did not hybridize to sections of uninfected mouse ganglia. Multiple slides of infected tissue (each containing sections through approximately 10 ganglia) were used to determine mean LAT$^+$ neuronal profiles per ganglionic section using a 1 mm graticule to assist counting when necessary (Table 4.1). By assessing several hundred ganglionic sections at each spinal segment statistically defensible accuracy was assured. Absolute LAT$^+$ neuron numbers were not calculated, first because there is dispute regarding correction factors required to generate this information

Figure 4.2

Photomicrographs (magnification x 384) showing detection of LATs by *in situ* hybridization in sensory ganglia of C57BL10 mice 23 days after infection with HSV-1 strain SC16. Ganglionic sections were hybridized to a DIG-labelled LAT riboprobe from pBS-0. Bound probe was detected with phosphatase-conjugated anti-DIG antibodies. LATs (black areas) were detected in the nuclei of primary sensory neurons at spinal segment T8 (A) and T12 (B). This range of staining intensity was typical and similar at all spinal segments (T8 to L1). Staining was not observed in sections of spinal ganglia from uninfected mice (C).


A


B


C

(Smolen *et al.*, 1983) and second because comparative data were sufficient in this context. In stark contrast to latent HSV DNA, LAT^+ neurons were readily detected in all spinal segments between T8 and L1, and there was no apparent difference in intensity of the LAT signal between segments (Figure 4.2 A,B).

4.1.3 Number of HSV DNA genomes per LAT^+ neuron

By comparing the number of HSV genome equivalents per cell with the number of LAT^+ neuronal profiles per ganglionic section, it was possible to estimate the number of latent viral genomes per LAT^+ neuron from spinal ganglia between T8-L1 (Table 4.1). This was achieved by firstly expressing the number of LAT^+ profiles/section as a percentage of the total neuronal profiles. The percentage of LAT^+ neuronal profiles/ganglionic section was calculated using an estimate of total neuronal profiles/ganglionic section of 128.4 $\pm$ 3.3 ($\pm$ standard error of the mean), derived from pooled ganglia from C57BL10 mice (D. Tscharke and A. Simmons, unpublished results). The number of viral genomes per LAT^+ neuron was then calculated on the basis that 10% of ganglionic cells are neurons (Walz *et al.*, 1976).

For example, at T8 there were assumed to be 128.4 neuronal profiles/ganglionic section of which 0.25/section (ie. 0.2%) were LAT^+. There were 0.056 HSV genomes/cell, but only 10% of cells were neurons ie. there were 0.56 HSV genomes/neuron. Thus, if 0.2% of neurons were LAT^+, then each LAT^+ neuron contained 0.56 x (100/0.2) HSV genomes ie. **280**.

When the number of copies of viral DNA detected at each spinal segment was related to the proportion of neurons containing LATs, two distinct patterns emerged. In ganglia that had little or no direct neural connection with the inoculation site (T10 to L1) there were approximately 20 HSV genome copies per LAT^+ neuron. In contrast, at T8 and T9, the abundance of viral DNA was at least 10-fold greater than at T10 to L1 in relation to each LAT^+ neuron (Table 4.1), and up to 50-fold greater in absolute terms.

The possibility of differences in neuronal numbers between spinal levels influencing estimates of viral DNA copy number/LAT^+ neuron is considered unlikely since studies on mammalian ganglia have shown neuronal numbers to vary by less than two-fold over a wide range of ganglionic levels (Hatai 1902; Holmes and Davenport, 1940).

4.1.4 Discussion

HSV DNA extracted from latently infected tissue has been quantified and structurally characterized previously (Rock & Fraser, 1983, 1985; Efstathiou *et al*, 1986). The molecules lack free ends, compatible with the presence of circular or concatemeric structures and they can be readily separated from chromosomal DNA, indicating that at least the bulk of the viral genomes are not integrated into host genetic material (Mellerick & Fraser, 1987). The amount of latent viral DNA recovered from the most abundantly infected C57BL10 ganglia (e.g. 0.056 copies/cell at T8) is similar to that recovered

from human trigeminal ganglia but several fold lower than that reported previously for BALB/c mice (Efstathiou *et al*, 1986), which may be due to the restrictive infection experienced by C57BL10 mice after flank inoculation (Simmons & LaVista, 1989). Nevertheless, in T8 and T9 ganglia, as in other systems, LAT^+ neurons would appear to contain hundreds of HSV DNA molecules. The results presented here show that many LAT^+ neurons, such as those between T10 and L1 in this experimental system, are able to maintain only a small number of HSV genomes, despite the presence of much higher DNA levels in ganglia innervating the inoculation site. There are three potential explanations of these data. First, LAT^+ neurons at T8 and T9 may contain more viral DNA than those at T10-L1, perhaps as a consequence of viral gene expression during the establishment phase (Speck & Simmons, 1991). In this context, circular and concatemeric DNA molecules are both thought to exist at different stages of the replicative cycle of HSV (Roizman & Sears, 1990). Second, LAT^+ neurons detected at T8 and T9 may represent a mixed population of cells, each of which contains either high (200+) or low (approximately 20) viral genomes. Finally, the bulk of the DNA recovered from the PNS of latently infected hosts (found at T8 and T9 in the system described here) may not reside in LAT^+ neurons. In these experiments the abundance of viral transcripts in neuronal nuclei (judged by staining intensity) appeared to be similar at all spinal segments tested, despite the potential difference in the number of DNA templates present. However, a direct comparison of viral DNA and RNA recovered from each spinal segment would

further characterize the apparent dissociation between transcriptional activity and DNA copy number (see section 4.2).

Using the standard technique of explant culture (Stevens & Cook, 1971) it has previously been shown, using the same experimental model, that latent HSV can be reactivated readily from all spinal segments between T6 and L1 despite a much more restricted acute infection, (Speck & Simmons, 1991), suggesting that LAT^+ neurons containing very few viral genomes are an important reservoir of infection. The paucity of HSV DNA in these cells is compatible with the lack of detectable viral gene expression in ganglia distant from those innervating the inoculation site during the acute phase of infection (Speck & Simmons, 1991) and the ability of replication incompetent mutant viruses to establish latency (eg. Leib *et al*, 1989). Limitations imposed by the sensitivity of ISH mean that the measurement of the proportion of neurons containing LATs (e.g. 0.34% at T12) must be regarded as the lower limit of the true figure, and therefore the actual number of HSV DNA molecules maintained in LAT^+ cells may be even lower than those calculated. Nevertheless, there is an intriguing similarity between the low copy pattern estimate (8-24 genomes/LAT^+ neuron at T10-L1) and the average number of subnuclear foci of minor LATs (13-14) detected in neuronal nuclei (Arthur *et al.*, 1993). It is becoming increasingly apparent that focal accumulations of primary RNA transcripts in cell nuclei represent sites of transcription (Xing *et al.*, 1993) and, in the case of EBV infected cells, foci of latent transcripts are thought to mark

the location of individual viral genomes (Xing and Lawrence, 1991). Further studies are required to determine whether a burden of the 8-24 genomes/LAT$^+$ neuron truly represents 'input' DNA or whether there is limited amplification of HSV DNA during or after the establishment phase, for instance by the interaction of cellular enzymes with viral genomes, as suggested by Sears and Roizman (1990).

The model system described here makes novel use of the segmental innervation of the thorax and abdomen. Spread of virus (via the 'back-door' route) to parts of the PNS adjacent to those innervating the inoculation site is well documented (Tullo *et al*, 1982). Although the factors that determine whether a neuron becomes productively or latently infected with HSV are poorly understood, careful manipulation of mouse strain, virus dose and site of inoculation enabled the selection of only a single outcome, latency, following spread of virus within the PNS. This system allows establishment and maintenance of latency to be characterized without the potential complications introduced by concurrent or resolved productive infection.

4.2 Distribution of latent HSV DNA, number of LAT$^+$ neurons and amount of LATs in corresponding spinal ganglia (T7-T12)

In an extension of the work presented in section 4.1, the apparent dissociation between viral DNA copy number and amount of LATs in latently infected spinal ganglia was examined more closely. Specifically, the distribution of latent HSV DNA was compared with the relative amount of LATs produced in ganglia from the same spinal segment, as well as the number of LAT$^+$ neurons. Adult animals were infected (day 0) with $3x10^4$ pfu/ml of HSV-1 strain SC16 by scarification of the left flank skin at the T7/T8 dermatome. On day 25 after infection, mice were killed and ganglia from spinal levels T7-T12 were removed for extraction of DNA (65 animals) or total RNA (70 animals). Another 4 mice were perfuse-fixed with PLP and their spines removed, decalcified, embedded in paraffin and prepared for *in situ* hybridization.

4.2.1 Quantification of latent HSV DNA by Southern blot hybridization

DNA samples from segmentally pooled ganglia (T7-T12) were digested with *Bam*HI and analysed by Southern blot hybridization using a ^{32}P-labelled probe (pBAZ-1) against the TK region of HSV-1. Bound probe was detected by phosphor imaging (20 hours exposure). In latently infected ganglionic DNA samples, the probe hybridized to a single 3.5 kb band corresponding to the *Bam*HI-Q fragment of HSV-1 strain SC16, and did not hybridize to cellular DNA from uninfected mice (Figure 4.3). Bands were quantified in relation to

Figure 4.3

Detection of HSV DNA sequences in latently infected spinal ganglia of C57BL10 mice 25 days after flank inoculation with HSV-1 strain SC16. Samples (10 μg) were digested with *Bam*HI, Southern blotted, probed with a ^{32}P-labelled DNA probe from pBAZ-1 and analysed by phosphor imaging. Distribution of HSV DNA (20 hour Phosphor Image): spinal segments T7 to T12 (lanes 4 to 9), reconstructions for quantification purposes using 0.5 and 0.05 HSV genome equivalents/cell (lanes 1 and 2, respectively) and uninfected mouse spleen DNA (lane 3). Arrowhead indicates the 3.5 kb *Bam*HI-Q fragment of HSV-1.


1 2 3 4 5 6 7 8 9

the 0.05 copy/cell reconstruction, using ImageQuant software (Table 4.2). HSV DNA was detected at all spinal segments examined (T7-T12) and was most abundant at T8.

4.2.2 Enumeration of LAT$^+$ neurons by *in situ* hybridization

In order to assess the relative number of LAT$^+$ primary sensory neurons at each spinal segment, coronal sections of spinal columns (Figure 4.4), rather than sequentially pooled ganglia, were analysed by *in situ* hybridization using a DIG-labelled LAT probe generated from pSLAT-4. The anatomical location of spinal ganglia was determined using the thirteenth thoracic vertebra, as a reference point, identified by its articulation with the lowest rib (which could be visualized in these sections). In infected tissue, LAT$^+$ neurons were detected at all spinal segments examined (left, T7-T12) and the intensity of staining was similar in all of these segments (eg. Figure 4.5 A,B). Multiple sections were used to determine the mean number of LAT+ neuronal profiles/ganglionic section (Table 4.2). The abundance of LAT$^+$ neurons was greatest at T9 and T10. The probe did not hybridize to coronal sections of spines from uninfected mice.

4.2.3 Semi-quantitative analysis of LATs by northern blot hybridization.

Using guanidine acetate buffer, total RNA was extracted from segmentally pooled ganglia at spinal segments T7, T8 and T9; for technical reasons, the amount of RNA recovered from T10-T12 was too small for analysis. Samples

TABLE 4.2 RELATIONSHIP BETWEEN HSV DNA, NUMBER OF LAT$^+$ NEURONS AND AMOUNT OF LATs IN SPINAL GANGLIA T7-T12

Spinal segment	HSV genome equivalents/ cell	LAT$^+$ profiles/section ± SEM (%)[a]	HSV genome equivalents/ LAT$^+$ neuron[b]	LATs detected by northern blot analysis (pixel no.)
T7	0.025	0.11 ± 0.06 (0.09)	278	1000
T8	0.066	0.69 ± 0.14 (0.54)	122	1900
T9	0.012	1.50 ± 0.29 (1.17)	10	3100
T10	0.024	1.52 ± 0.21 (1.18)	20	NT
T11	0.018	1.23 ± 0.16 (0.96)	19	NT
T12	0.017	0.95 ± 0.17 (0.74)	23	NT

a The percentage of LAT$^+$ neuronal profiles/ganglionic section was calculated using an estimate of total neuronal profiles per ganglionic section of 128.4 ± 3.3 (± SEM). This estimate was derived from pooled thoracic ganglia from C57BL10 mice (D Tscharke and A Simmons, unpublished results).

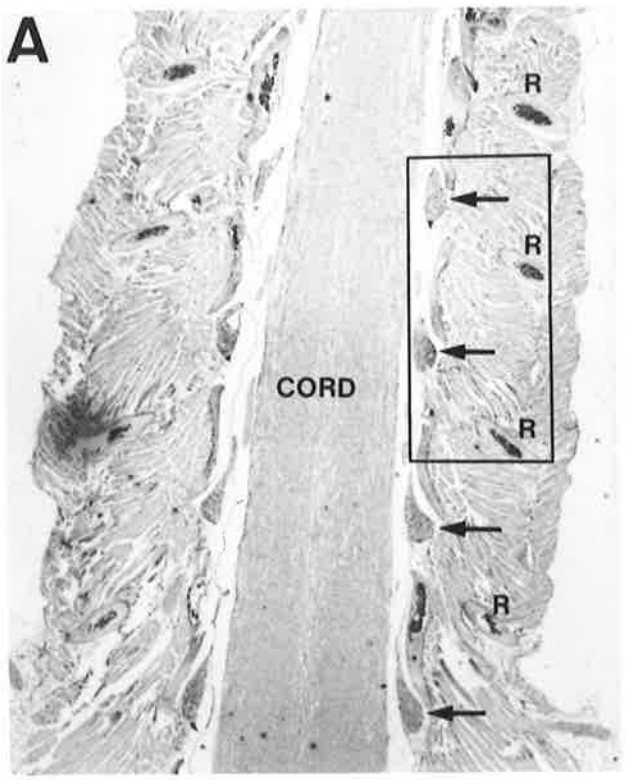
b The number of genomic equivalents/LAT$^+$ neuron was calculated by assuming that 10% of ganglionic cells are neurons (Walz *et al*, 1976).

NT Not tested.

Figure 4.4

(A) Photomicrograph (magnification x 7.5) of a coronal section of the lower thoracic region of the spinal column of an uninfected C57BL10 mouse. The spinal cord (cord), dorsal root ganglia (arrows) and ribs (R) are illustrated.

(B) Higher magnification (x 20) of (A) showing the 11th (T11) and 12th (T12) thoracic ganglia and associated ribs (R).


A
R
R
CORD
R
R

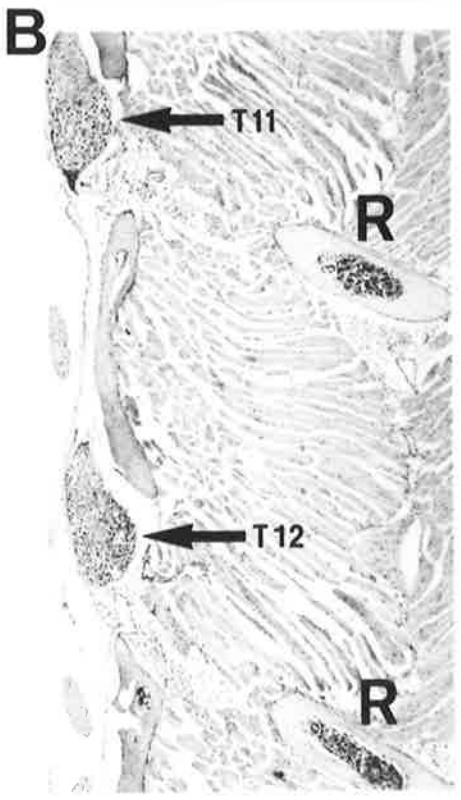
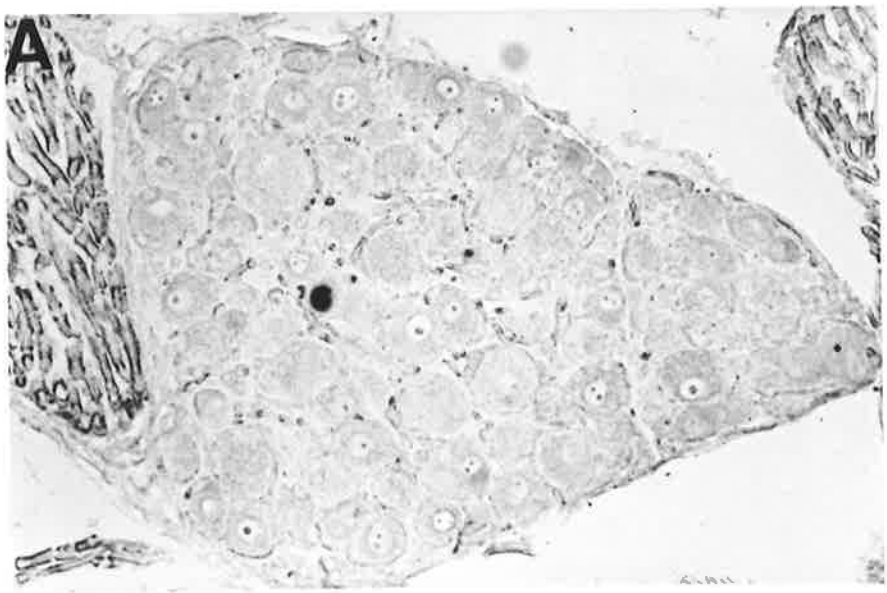
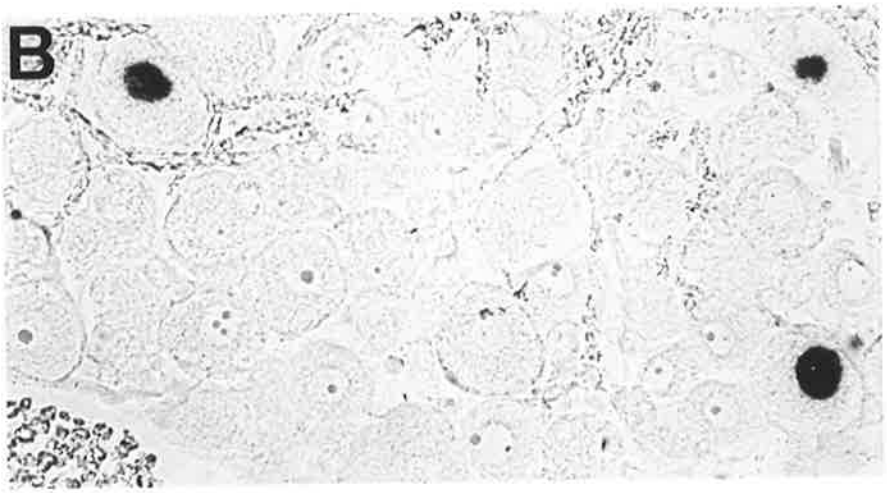
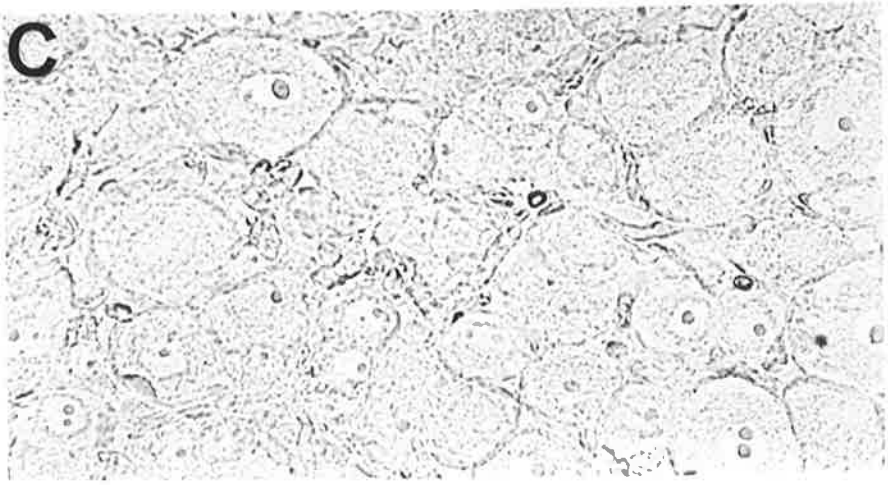

B
T11
R
T12
R

Figure 4.5

Detection of LATs by *in situ* hybridization in latently infected spinal ganglia of C57BL10 mice 25 days after flank inoculation with HSV-1 strain SC16. Coronal spinal sections were hybridized to a DIG-labelled LAT riboprobe from pSLAT-4. Bound probe was detected by phosphatase-conjugated anti-DIG antibodies. Photomicrographs of ganglionic sections from T7 (A; magnification x 96) and T9 (B; magnification x 300) showing LAT staining (black areas) in neuronal nuclei. This staining intensity was typical and similar at all spinal segments (T7 to T12). Staining was not observed in sections from uninfected mice (C; magnification x 300).


A


B


C

(T7-T9) were analysed by northern blot hybridization using a cloned ^{32}P-labelled DNA probe generated from plasmid pSLAT-4. Bound probe was detected after exposure to a phosphor screen for 20 hours (Figure 4.6 A). In all spinal segments examined (T7-T9), the probe hybridized to a single 2.0 kb band corresponding to the 2.0 kb major LAT species. Hybridization to RNA extracted from the ganglia of uninfected mice was not observed. After reprobing filters for the transcripts of a cellular housekeeping gene, mouse β-actin (Alonso *et al*, 1986), it was found that the loading of total RNA in the T7 sample was higher than that of T8 and T9 (Figure 4.6 B). Subsequently, all ImageQuant values of LAT band intensity were normalized to take into account loading variability. Results were expressed as pixel counts per band (Table 4.2), enabling comparison of the relative amounts of LATs at each spinal level. It was concluded that the 2.0 kb major LAT species was more abundant at T9 than at T7 or T8.

4.2.4 Relationship between HSV DNA, number of LAT$^+$ neurons and amount of LATs in spinal ganglia T7 to T12

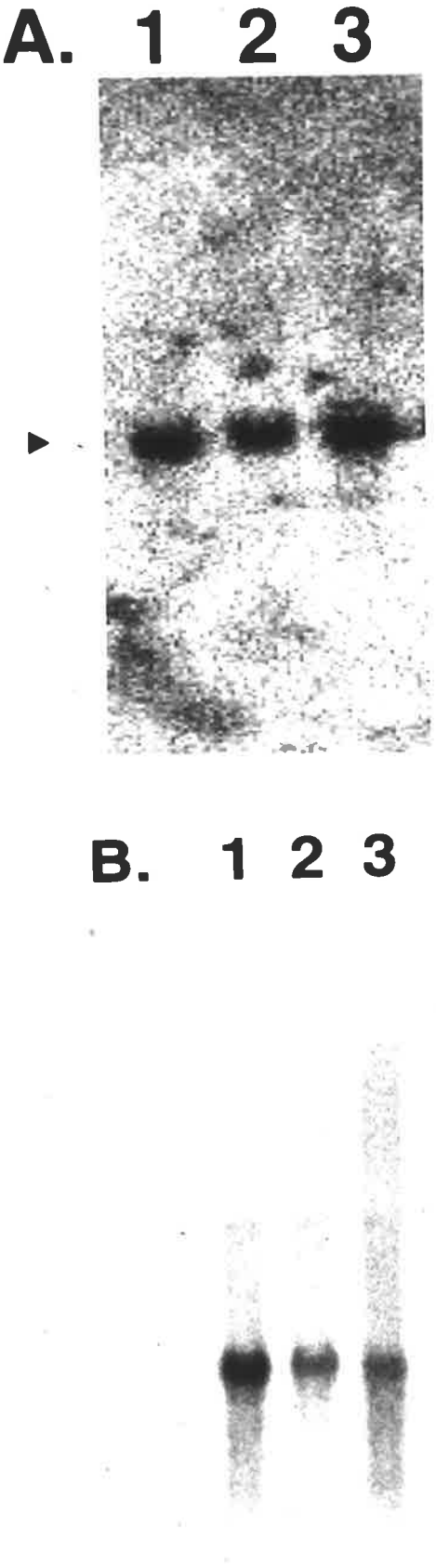
Comparison between the number of copies of viral DNA and the number of LAT$^+$ neurons in corresponding spinal segments disclosed two patterns of viral persistence, as reported in section 4.1. In ganglia from spinal segments T9-T12 there were 10-23 (average, 18) HSV genomes/LAT$^+$ neuron, whereas in segments T7 and T8, which innervated inoculated skin, there were between 122 and 278 (average, 200) genomes/LAT$^+$ neuron (Table 4.2).

Figure 4.6

Northern blot and Phosphor Image analysis of RNA from latently infected spinal ganglia of mice 25 days after flank inoculation with HSV-1 strain SC16.

(A) Phosphor Image at 20 hours showing the distribution of HSV LATs in samples from spinal segments T7 to T9 (lanes 1 to 3). Nitrocellulose filters were hybridized to a ^{32}P-labelled DNA probe derived from pSLAT-4. Arrowhead indicates the position of the 2.0 kb major LAT species.

(B) Reprobe of (A) with a ^{32}P-labelled DNA probe derived from pAL41 showing, for normalization purposes, the distribution of a cellular β-actin transcript in ganglionic samples from spinal segments T7 to T9 (lanes 1 to 3)


A.
1
2
3
B.
1
2
3

The number of HSV genomes/LAT$^+$ neuron in segments T7-T9 was compared with the relative amount of LATs recovered from corresponding sites (Table 4.2). At T9 the low number of genomes/LAT$^+$ neuron (10) corresponded to the highest yield of LATs, whereas at T7 and T8 there were 28- and 12-fold more genomes/LAT$^+$ neuron respectively, but fewer viral transcripts. It was concluded that the amount of viral DNA does not reflect the number of LAT$^+$ neurons or the amount of LATs in corresponding ganglia of latently infected mice.

4.2.5 Discussion

This study confirmed and extended the results discussed in the previous section (4.1). As before, the segmental sensory innervation of mouse flanks was utilized to demonstrate the presence of two patterns of viral DNA persistence which were distinguished by a high (average, 200, at T7/T8) and low (average, 18, at T9-T12) number of genomes/LAT$^+$ neuron. The high copy number latency was associated, anatomically, with viral replication during the acute phase of infection. The potential explanations for these patterns of viral persistence have already been discussed (see section 4.1). Comparison of the number of HSV genomes/LAT$^+$ neuron with the relative amount of LATs recovered from corresponding sites reinforced the clear dissociation between DNA copy number and transcriptional activity in this system. A small number of viral genomes/LAT$^+$ neuron (at T9) accounted for the highest yield of LATs. Thus, the transcriptional activity of the bulk of the DNA recovered

from ganglia (ie. at T7 and T8) after infection with a virulent HSV, and its location, remain uncertain. The data presented here strongly imply that most viral DNA sequences that persist in the PNS may be transcriptionally silent, perhaps resembling the repressed nature of the viral genome in autonomic ganglia (Rødahl and Stevens, 1992). This conclusion is supported by the observation that LATs accumulate to wild-type levels under conditions that preclude amplification of the viral genome (Tenser *et al.*, 1989; Valyi-Nagy *et al.*, 1991). An alternative explanation of the data might be that the level of transcription from each latent viral genome may be greatly reduced when a large number (*circa* 200) of genomes are present in a single cell.

5. INFECTION OF SPINAL GANGLIA WITH A RECOMBINANT HSV DEFICIENT IN ITS ABILITY TO EXPRESS THYMIDINE KINASE

Based on the data presented in the previous chapter, it was hypothesized that the bulk of the DNA recovered from ganglia latently infected with virulent HSV-1 strain SC16 is generated by replication of virus in neurons during the establishment phase. To address this hypothesis, mice were infected with a strain of virus that cannot replicate in the PNS and ganglionic infection was characterized in terms of HSV DNA stability, structure and genome copy number/LAT$^+$ neuron. A well characterized thymidine kinase (TK) deletion mutant, TKDM21 (Efstathiou *et al.*, 1989), which replicates in skin but not in the nervous system, was selected for this study.

5.1 Characterization of HSV-1 TKDM21 in spinal ganglia at early times after cutaneous infection

In previous studies, detection of HSV-1 TKDM21 DNA in spinal ganglia of mice experimentally infected in the ear pinna has been problematic, presumably because TKDM21 cannot replicate in the PNS (Efstathiou *et al.*, 1989). In the present study, the experimental system was modified in two ways to maximize the number of neurons infected with HSV. Firstly, in order to facilitate direct quantification of TKDM21 DNA, the inoculation site was

extended to include four mid-flank dermatomes (T8-T11) and secondly, the dose of inoculum was increased from 3 x 10^4 pfu/mouse to 3 x 10^7 pfu/mouse.

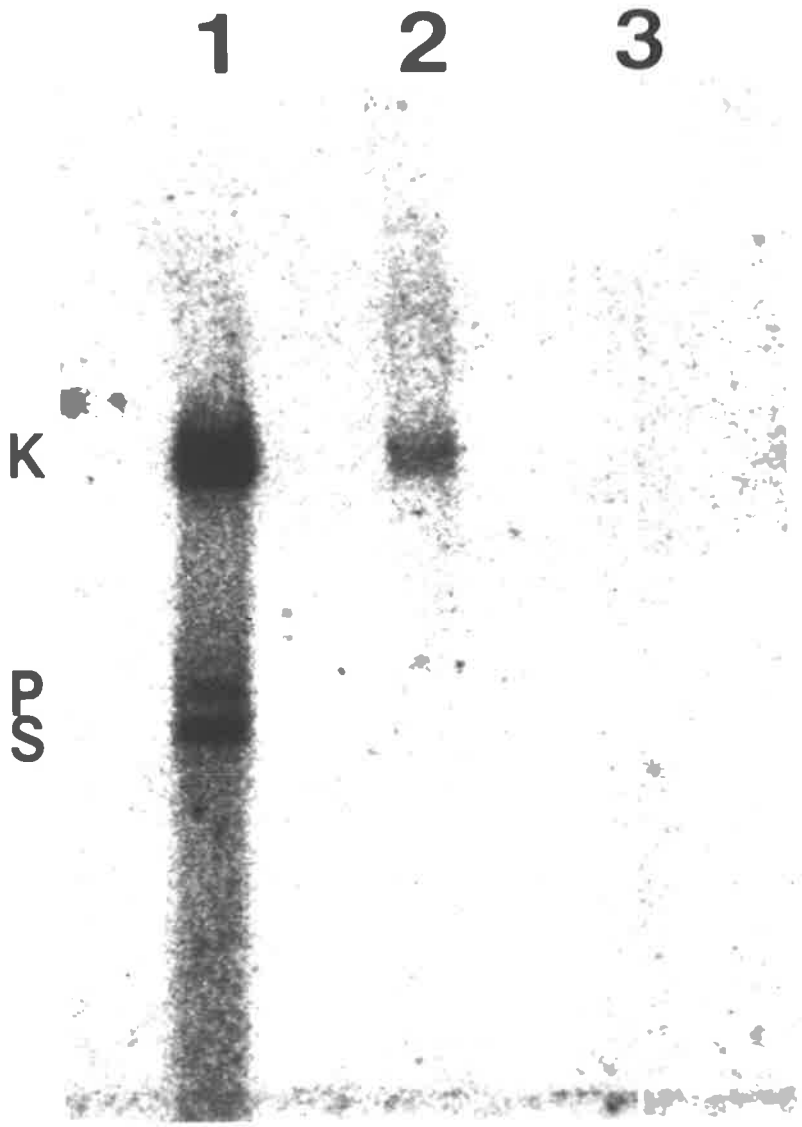
5.1.1 Structural comparison of SC16 and TKDM21 genomes in spinal ganglia 5 days after infection

Twelve C57BL10 mice were infected (day 0) by scarification of the left flank at the T8 dermatome with 3 x 10^4 pfu HSV-1 strain SC16. On day 5, ganglia (left, T8-T11) were pooled and tested for the presence of HSV DNA. A second group of 10 mice were inoculated (day 0) across 4 dermatomes (left, T8-T11) with 3 x 10^7 pfu HSV-1 strain TKDM21. On day 5, ganglia spanning the inoculation site (left, T8-T11) were pooled and tested for the presence of HSV DNA.

Ganglionic DNA from SC16 infected animals was diluted 1:7 in uninfected ganglionic DNA prior to analysis in order to reduce band intensity. Samples (10 μg) were digested with *Bam*HI and analysed by Southern blot hybridization using a ^{32}P-labelled probe from pBKSP1, which spans the junction region of HSV-1. Bound probe was detected by phosphor imaging (24 hour exposure) (Figure 5.1). In SC16 infected ganglionic samples, the probe hybridized to 3 bands of 5.9 kb, 3.6 kb and 2.9 kb, corresponding to the expected sizes of the *Bam*HI-K (junction), *Bam*HI-P (terminal) and *Bam*HI-S (terminal) viral fragments, repectively. In TKDM21 infected samples, the probe detected a 5.9 kb band only, corresponding to the *Bam*HI-K junction fragment. ie. viral

Figure 5.1

Detection of HSV DNA sequences in spinal ganglia 5 days after infection with either HSV-1 strain SC16 (lane 1) or HSV-1 strain TKDM21 (lane 2) by Southern blot hybridization and Phosphor Image analysis (24 hour exposure). DNA samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose and hybridized with a ^{32}P-labelled junction probe derived from pBKSP1. The junction (K; 5.9 kb) and termini (P; 3.6kb and S; 2.9 kb) of HSV-1 strain SC16 are indicated. Note the lack of free genomic termini in ganglia from TKDM21 infected animals. Lane 3 contains uninfected mouse spleen DNA.


1
2
3
K
P
S

genomic termini were not detected. The probe did not hybridize to DNA extracted from uninfected mice. It was concluded that 5 days after infection TKDM21 genomes were in a structurally different configuration to SC16 genomes.

5.1.2 Analysis of spinal ganglia 3 and 5 days after cutaneous infection with TKDM21

The previous result implied that TKDM21 linear virion DNA undergoes a structural alteration compatible with interuption of replication after circularization of input genomes. To confirm that TKDM21 does not replicate in spinal ganglia, viral DNA and infectious virus were examined 3 and 5 days after infection. C57BL10 mice were infected (day 0) by scarification across 4 dermatomes (left, T8-T11) with 3 x 10^7 pfu HSV-1 strain TKDM21. On day 3 and day 5, ganglia innervating the inoculation site (left, T8-T11) were pooled and tested for the presence of HSV DNA (10 animals/day) or infectious virus (3 mice/day). A second group of mice were infected (day 0) by scarification at the left,T8 dermatome with 3 x 10^7 pfu HSV-1 strain SC16. On day 3 and day 5, ganglia spanning T8-T11 were pooled and tested for the presence of infectious virus (1 mouse/day).

5.1.2.1 *Semi-quantitative structural analysis of TKDM21 genomes*

Ganglionic DNA samples (10 μg) were digested with *Bam*HI and analysed by Southern blot hybridization using a ^{32}P-labelled DNA probe derived from pBKSP1. Bound probe was detected by phosphor image analysis (24 hour exposure) (Figure 5.2). The probe bound to a 5.9 kb band, corresponding to the *Bam*HI-K junction fragment of HSV-1. Terminal fragments (*Bam*HI-P and *Bam*HI-S) were not detected. The intensity of the junction band detected on days 3 and 5 was assessed using ImageQuant software and expressed as pixel counts/band. Junction band intensity was comparable on day 3 (1.9 x 10^6 pixels) and day 5 (1.7 x 10^6 pixels). In samples (10 μg) containing dilutions of SC16 virion DNA, probe hybridized to 3 bands corresponding to the predicted sizes of the junction (*Bam*HI-K) and terminal (*Bam*HI-P and *Bam*HI-S) fragments of the viral genome. The probe did not hybridize to DNA extracted from uninfected mice. It was concluded that the amount of TKDM21 DNA recovered from spinal ganglia was stable between day 3 and day 5. As in the previous experiment (section 5.1.1), TKDM21 DNA lacked free genomic termini.

5.1.2.2 *Quantification of infectious virus in spinal ganglia.*

To determine the amount of infectious virus in ganglia 3 and 5 days after infection, homogenized samples were tested by plaque assay on Vero cell monolayers (Figure 5.3). Homogenates were analysed at a starting dilution of 1:2. In ganglia from mice infected with HSV-1 strain SC16 there were 5.5 x

Figure 5.2

Detection of HSV *Bam*HI-K junction fragment in DNA extracted from spinal ganglia (pooled T8 to T11) of C57BL10 mice 3 days (lane 5) and 5 days (lane 6) after cutaneous inoculation with HSV-1 strain TKDM21. Lanes 1 to 3 represent 1:10 serial dilutions of HSV-1 virion DNA in uninfected mouse spleen DNA. Lane 7 is DNA extracted from the spleens of uninfected mice. Samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose and hybridized with a ^{32}P-labelled probe derived from pBKSP1, and examined by phosphor imaging (24 hour exposure). The position of the junction (K) and terminal fragments (P and S) are indicated.

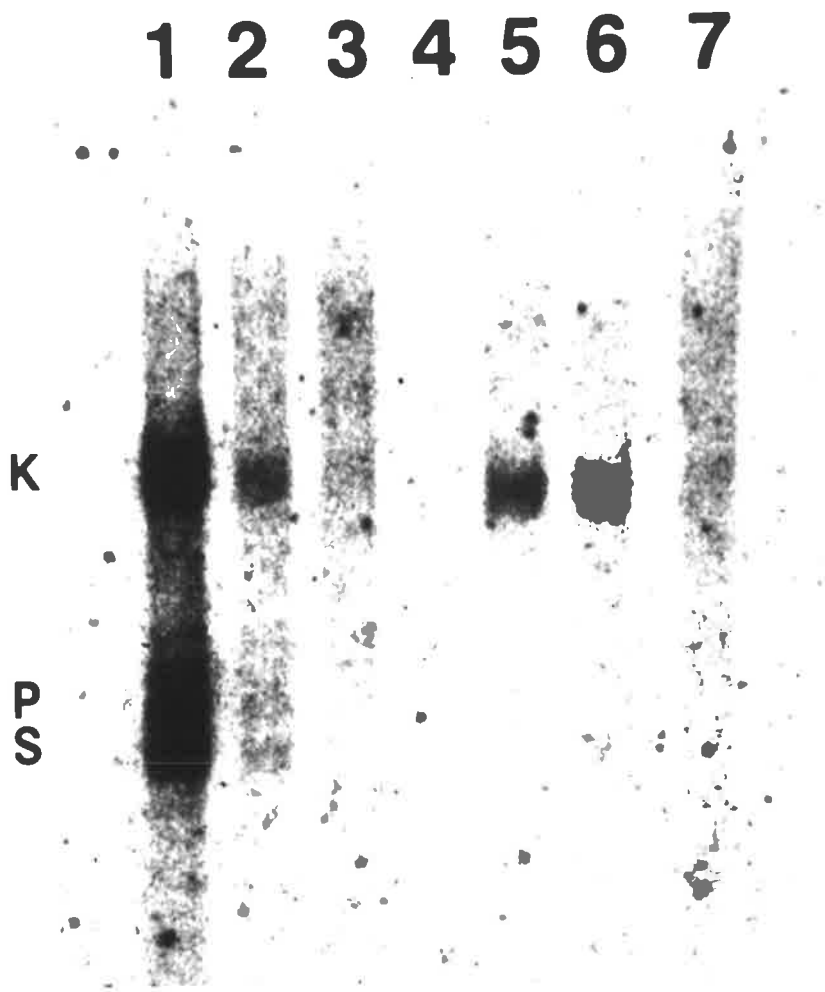
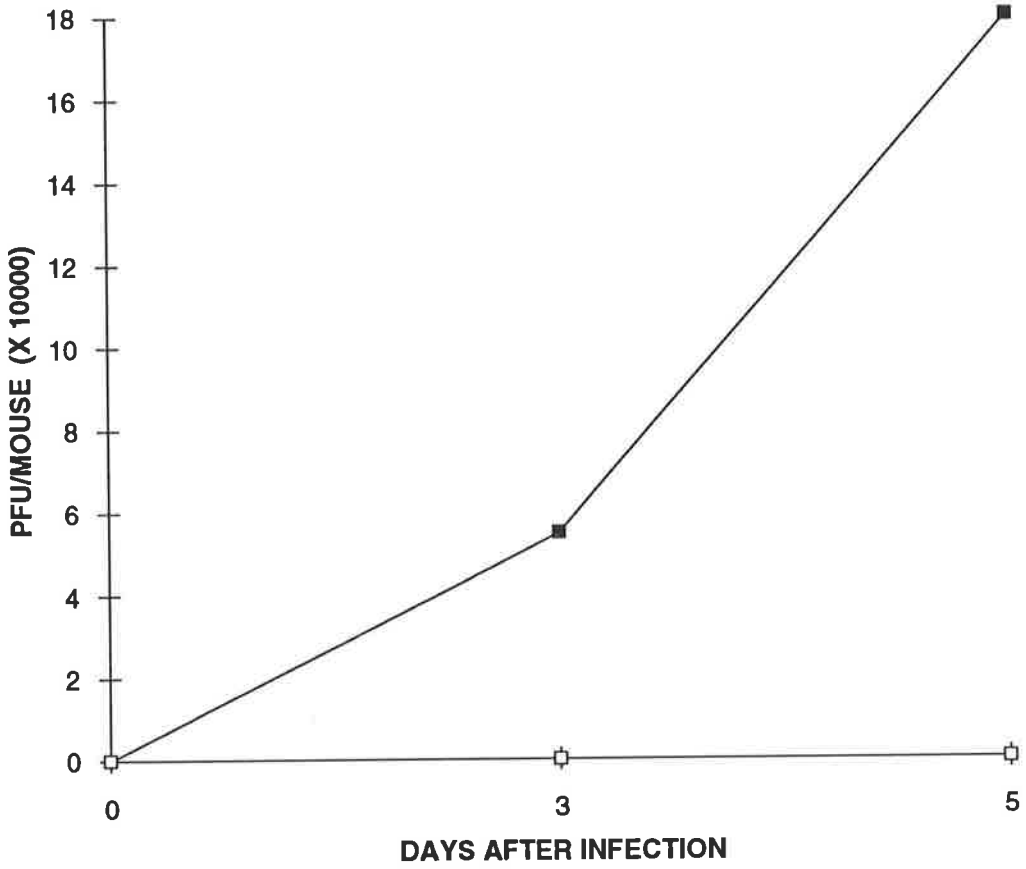

1 2 3 4 5 6 7
K
P
S

Figure 5.3

Quantification of infectious HSV in spinal ganglia (pooled T8 to T11) 3 and 5 days after cutaneous infection with HSV-1 strain SC16 (solid squares) and HSV-1 strain TKDM21 (open squares). Ganglionic homogenates from TKDM21 infected animals contained < 2 pfu/mouse on day 3 and day 5.

Figure 5.3


18
16
14
12
10
8
6
4
2
0
PFU/MOUSE (X 10000)
0
3
5
DAYS AFTER INFECTION

10^4 and 1.8 x 10^5 pfu/mouse on day 3 and day 5, respectively. In contrast, infectious virus was not detected in either the day 3 or day 5 samples from mice infected with HSV-1 strain TKDM21. It was concluded that inoculation of C57BL10 mice with HSV-1 strain TKDM21 does not result in detectable productive ganglionic infection.

5.2 Number of latent HSV-1 TKDM21 genomes per LAT$^+$ neuron in spinal ganglia innervating the site of virus inoculation

The experiments in this section sought to investigate the hypothesis that the bulk of the viral DNA recovered from ganglia latently infected with virulent HSV-1 strain SC16 (see chapter 4) is derived from amplification of viral DNA during the acute phase of infection. It was reasoned that establishment of latency with TKDM21, which is replication defective in ganglia, should reduce the number of viral genomes/LAT$^+$ neuron, in ganglia directly innervating the site of inoculation, from the high copy pattern (approximately 200) to that of the low copy pattern (8-24, as is seen in ganglia not innervating the inoculation site after SC16 infection). C57BL10 mice were inoculated across four dermatomes (left, T8-T11) with 3 x 10^7 pfu of HSV-1 strain TKDM21. Thirty two days after infection, ganglia innervating the inoculation site (left, T8-T11) were pooled and tested for the presence of HSV DNA (18 animals) or LAT$^+$ neurons (7 animals).

5.2.1 Quantification of TKDM21 DNA in latently infected spinal ganglia

Ganglionic DNA samples (10 μg) were digested with *Bam*HI and analysed by Southern blot hybridization using a ^{32}P-labelled probe generated from pBKSP1, which spans the junction region of HSV-1. Positive hybridization was detected after 18 hour exposure to phosphor screens. In TKDM21 infected ganglionic samples, probe hybridized to a single 5.9 kb band, corresponding to the *Bam*HI-K junction fragment of HSV-1 (Figure 5.4 A). The probe also hybridized to three bands of 5.9 kb, 3.6 kb and 2.9 kb, corresponding to the *Bam*HI-K, *Bam*HI-P and *Bam*HI-S fragments, respectively, in a positive control sample extracted from acutely infected (with TKDM21) Vero cells. Hybridization to DNA extracted from uninfected mice was not observed.

Samples (10 μg) were also analysed with a ^{32}P-labelled DNA probe generated from pSB-6, which contains the *Bam*HI-B fragment of HSV-1. The probe detected two bands of approximately 10.1 kb and 8.9 kb, corresponding to the expected sizes of the *Bam*HI-B and *Bam*HI-E fragments of HSV-1 (Figure 5.4 B). Hybridization to the same sized two fragments was detected in virion DNA 'copy/cell' reconstructions. The probe did not hybridize to uninfected mouse DNA.

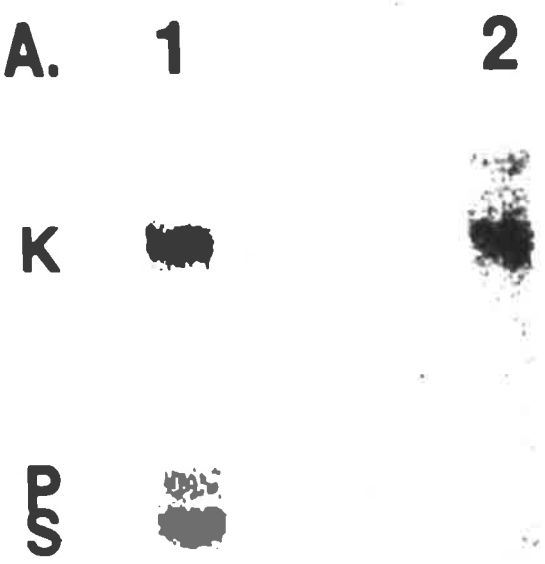
The amount of viral DNA in TKDM21 infected ganglionic samples was determined by comparing band intensity to that of the 0.05 copy/cell reconstruction using ImageQuant software. In these ganglia, all of which had

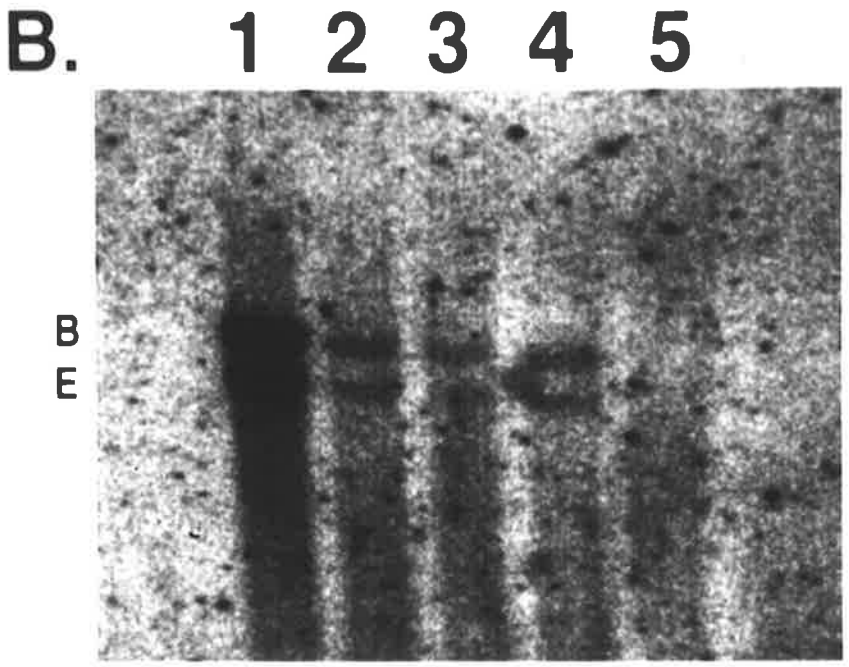
Figure 5.4

Detection of HSV DNA in latently infected spinal ganglia 32 days after flank inoculation with HSV-1 strain TKDM21 by Southern blot hybridization and phosphor imaging. Samples (10 μg) were digested with *Bam*HI, transferred to nitrocellulose and hybridized to HSV specific ^{32}P-labelled probes derived either from pBKSP1 (A) or pSB-6 (B).

(A) Phosphor Image at 18 hours showing the presence of HSV DNA sequences in samples probed with the junction probe pBKSP1. Viral junction (K; 5.9 kb) and terminal (P; 3.6 kb and S; 2.9 kb) fragments were observed in a DNA sample from Vero cells productively infected with HSV-1 TKDM21 (1:100 dilution made in 10 μg uninfected mouse spleen DNA)(lane 1). A band corresponding to the viral junction fragment of (K) was detected in TKDM21 infected ganglia (lane 2), but terminal fragments were absent.

(B) Phosphor image at 18 hours showing the presence of HSV DNA sequences in samples probed with the *Bam*HI-B probe pSB-6. The HSV-1 *Bam*HI-B (B; 10.1 kb) and *Bam*HI-E (E; 8.9 kb) fragments detected in TKDM21 infected ganglia (lane 3) and in reconstructions using 0.5 genome copies/cell (lane 1) and 0.05 HSV genome copies/cell (lanes 2 and 4) are indicated. Lane 5 is uninfected mouse spleen DNA.


A.
1
2
K
PS


B.
1 2 3 4 5
B
E

direct neural connections with the inoculation site, there were 0.034 HSV genomes/cell.

5.2.2 Enumeration of LAT$^+$ neurons in latent infected spinal ganglia

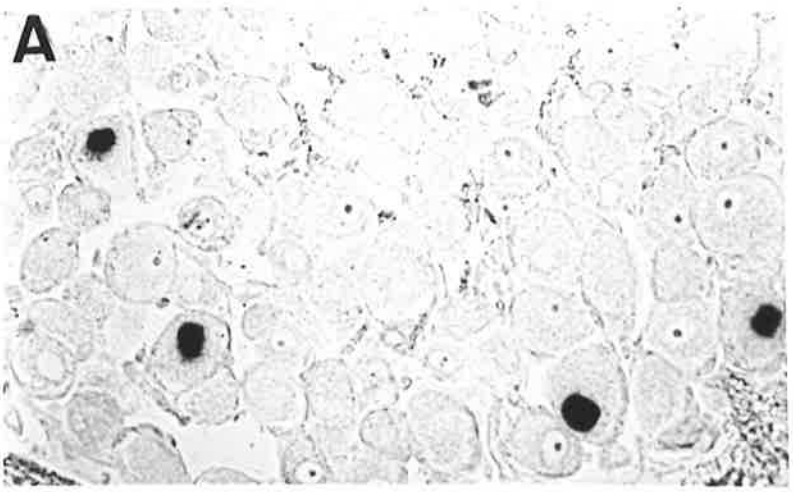
To enumerate LAT$^+$ neurons, ganglia were fixed in PLP, paraffin embedded and 5 μm sections were analysed by *in situ* hybridization using a DIG-labelled LAT riboprobe from pSLAT-4. The probe hybridized strongly to neurons in ganglia of mice latently infected with TKDM21 and the intensity of staining was indistinguishable from that observed in ganglionic sections from mice latently infected with HSV-1 strain SC16 (Figure 5.5). Hybridization was not observed in ganglia from uninfected animals. After analysis of 262 ganglionic sections, the mean number of LAT$^+$ neuronal profiles/ganglionic section was calculated to be 1.97 $\pm$ 0.14 ($\pm$ S.E.M.)

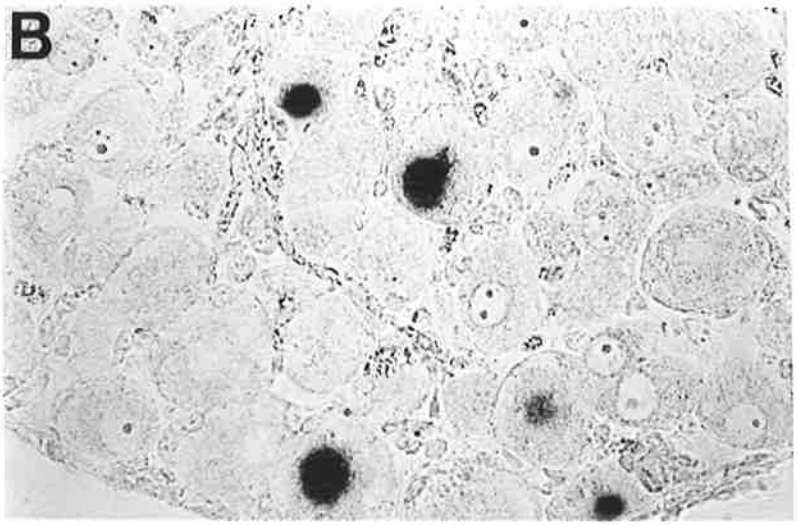
5.2.3 Number of TKDM21 genomes/LAT$^+$ neuron

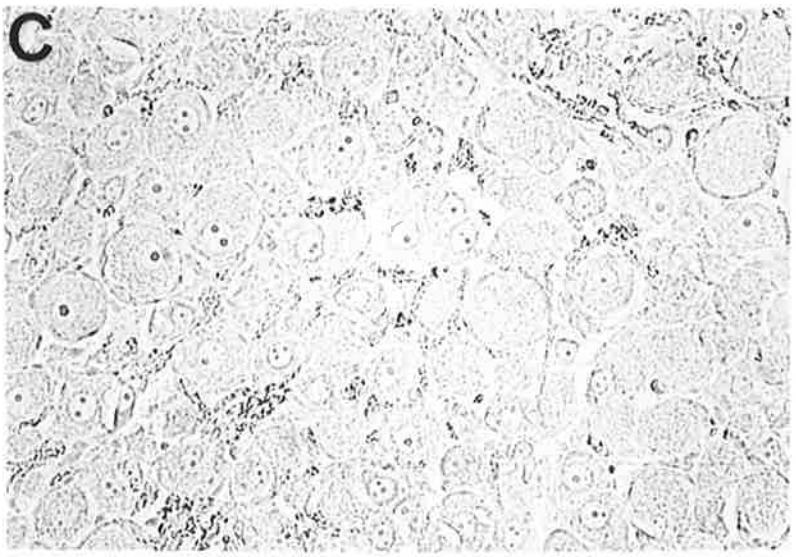
An estimate of the number of HSV TKDM21 genomes/LAT$^+$ neuron was made by comparing the number of viral genomes/cell with the number of LAT$^+$ neuronal profiles/ganglionic section. As in the previous chapter this calculation assumed that (i) 10% of ganglionic cells are neurons (Walz *et al.*, 1976) and (ii), the average total number of neuronal profiles/ganglionic section is 128.4 (D. Tscharke and A. Simmons, unpublished data). It was estimated that each LAT$^+$ neuron contained 22 HSV-1 strain TKDM21 genomes.

Figure 5.5

Detection of LATs (black areas) by *in situ* hybridization in latently infected spinal ganglia of C57BL10 mice 32 days after flank inoculation with HSV-1 strain TKDM21 (A; magnification x 300) and 64 days after flank inoculation with the parental HSV-1 strain SC16 (B; magnification x 384). LAT staining was not observed in ganglionic sections from uninfected mice (C; magnification x 300). Sections were hybridized to a DIG-labelled LAT riboprobe derived from pSLAT-4. Bound probe was detected using phosphatase-conjugated anti-DIG antibodies.


A


B


C

5.2.4 Detection of LATs in neuronal cytoplasm

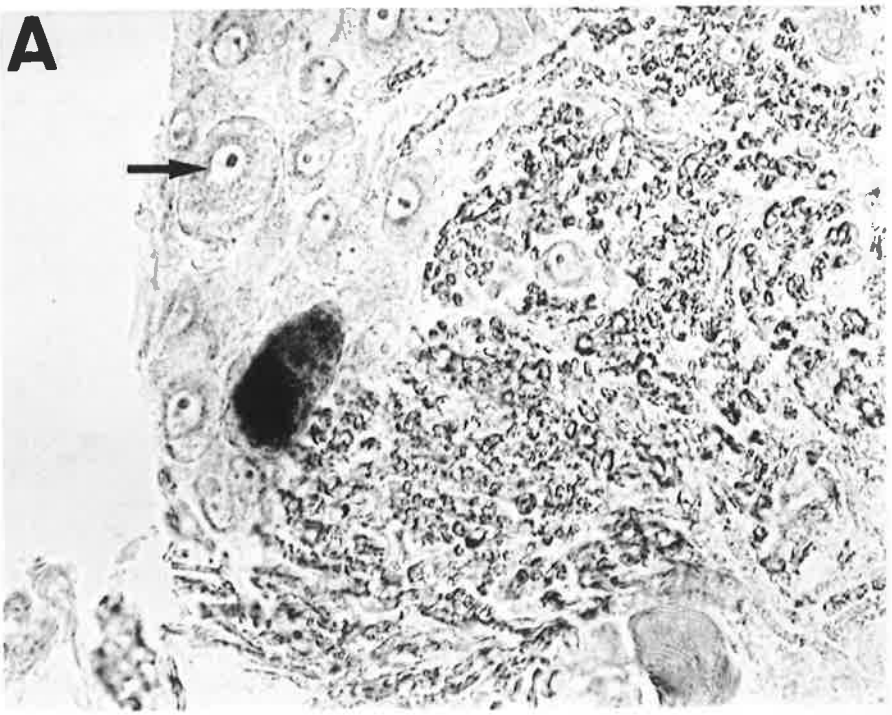
The *in situ* hybridization experiments described in this chapter included ganglionic sections from C57BL10 mice latently infected with HSV-1, strain SC16 (ganglia removed 64 days after infection with 2 x 10^7 pfu). In concordance with previous observations, LATs were in general localized to neuronal nuclei. However, both in SC16 and TKDM21 infected ganglia staining for LATs was not exclusively nuclear; rather, in a small proportion (<1%) of LAT^+ neurons staining was both nuclear and cytoplasmic (Figure 5.6). In some cells, cytoplasmic staining appeared as sharply defined foci whereas in other cells staining was diffuse, often filling the entire cytoplasm (Figure 5.7). Under high power magnification, foci of cytoplasmic LATs were visible against a background of diffuse staining. Staining was not observed in ganglia from uninfected mice. To determine whether viral antigens (associated for instance with spontaneous reactivation) could be detected in SC16 infected ganglia containing cytoplasmic LATs, a total of 123 ganglionic sections were analysed by immunohistochemistry, using a mouse anti-HSV polyclonal antibody. Viral antigen staining was not detected in any of the sections analysed. It was concluded that in latently infected ganglia, a small proportion of LAT^+ neurons display LAT specific staining in the cytoplasm as well as the nucleus.

Figure 5.6

Detection of LATs by *in situ* hybridization in the cytoplasm of primary sensory neurons from latently infected C57BL10 mice. Ganglionic sections were hybridized to a DIG-labelled LAT riboprobe derived from pSLAT-4. Bound probe was detected using phosphatase-conjugated anti-DIG antibodies.

(A) Photomicrograph (magnification x 300) of a ganglionic section 32 days after infection with HSV-1 strain TKDM21, showing LAT staining (black areas) in both the nucleus and cytoplasm of a primary sensory neuron. The nucleus of a LAT negative neuron is arrowed.

(B) Photomicrograph (magnification x 300) of a ganglionic section 64 days after infection with HSV-1 strain SC16, showing characteristic LAT staining (black areas) in the nucleus of primary sensory neurons, as well as cytoplasmic staining in another (arrow).


A

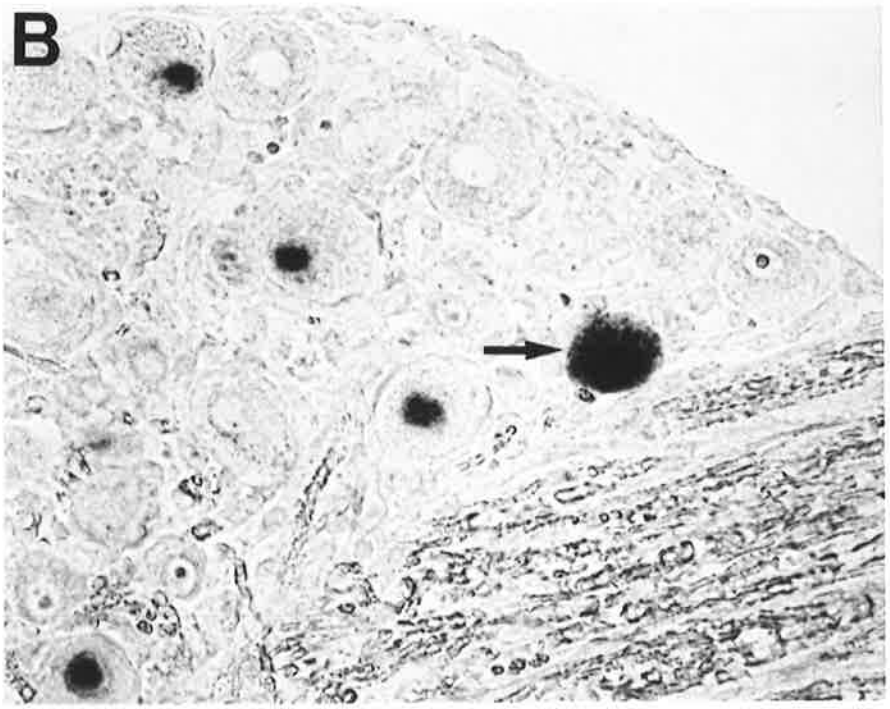

B

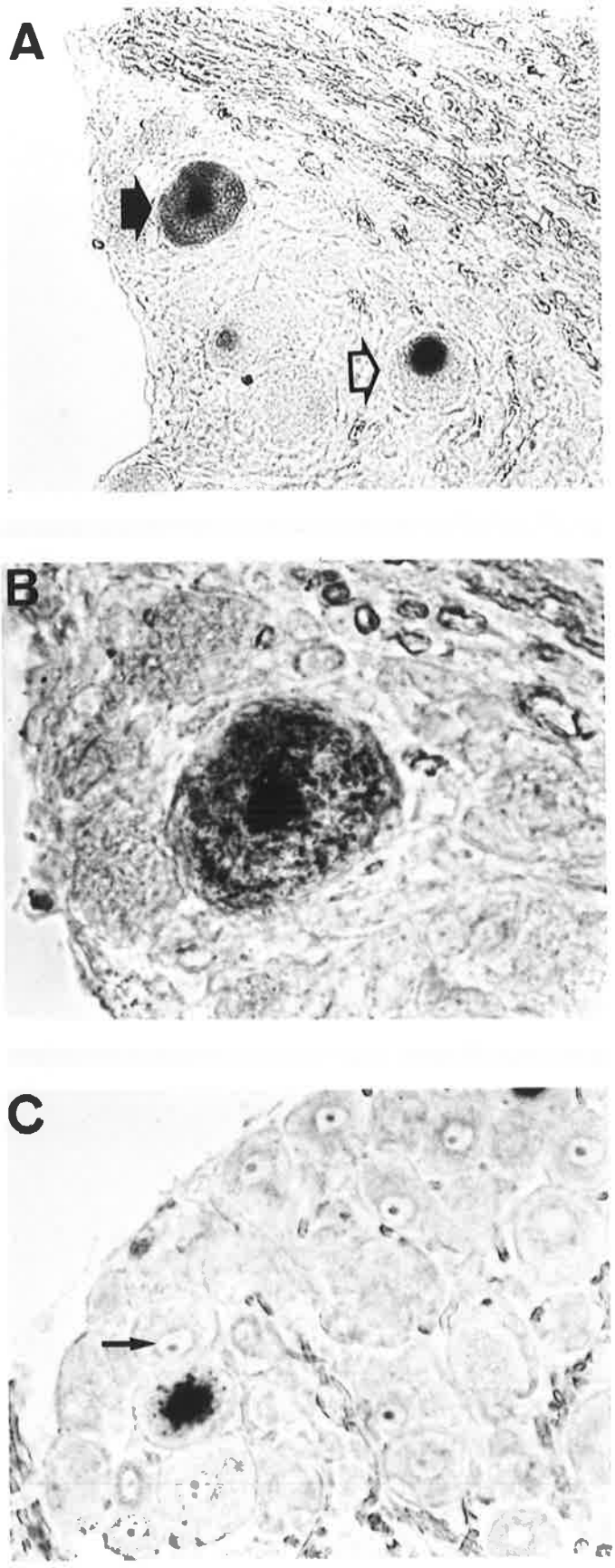
Figure 5.7

Photomicrographs showing detection of cytoplasmic LATs (black areas) by *in situ* hybridization, in latently infected spinal ganglia of C57BL10 mice 64 days after flank inoculation with HSV-1 strain SC16. Sections were hybridized to a DIG-labelled LAT riboprobe derived from pSLAT-4. Bound probe was detected using phosphatase-conjugated anti-DIG antibodies.

(A) Photomicrograph (magnification x 300) showing nuclear LAT staining in a primary sensory neuron (open arrow), and both nuclear and cytoplasmic LAT staining in another (solid arrow).

(B) Photomicrograph (magnification x 750) from (A) showing nuclear and cytoplasmic LAT staining (black areas) of a primary sensory neuron. Note the presence of LAT foci against a background of diffuse staining in the cytoplasm.

(C) Photmicrograph (magnification x 300) showing nuclear and cytoplasmic LAT staining (black areas) of a primary sensory neuron. In this cell LAT staining in the cytoplasm was confined to sharply defined foci. The arrow shows the nucleus of a LAT negative neuron.


A
B
C

5.3 Discussion

TKDM21 was shown previously to replicate almost as efficiently as parental SC16 in skin and to establish latency in sensory ganglia (judged by superinfection of explanted ganglia with wild-type virus) (Efstathiou *et al.*, 1989). In the same study, replication could not be detected in the nervous system, suggesting that virus-encoded TK activity is an essential requirement for replication in non-dividing cells. The protocol used to infect mice with TKDM21 in the experiments described in this chapter allowed TKDM21 genomes in spinal ganglia to be detected and analysed directly by Southern blot hybridization. This is the first report of the direct analysis of a replication defective HSV in the nervous system. The 'endless' nature of TKDM21 genomes detected 5 days after infection, the comparable amounts of viral DNA in ganglia on days 3 and 5, and the inability to detect infectious virus at these times led to the conclusion that TKDM21 DNA does not replicate during establishment of latency. In contrast, wild-type virus (SC16) was shown both here and previously (Speck and Simmons, 1991) to replicate in ganglia 3-5 days after cutaneous infection. Therefore, ganglionic infection with TKDM21 provided a means by which unamplified latent viral genomes could be studied, without interference from products of viral DNA replication.

In ganglia latently infected with TKDM21 (day 32 after infection), all of which directly innervated the inoculation site, there were approximately 22 viral genomes/LAT$^+$ neuron. This was in stark contrast to the average number of latent viral genomes/LAT$^+$ neuron (approximately 200) in ganglia directly innervating the inoculation site of mice infected with strain SC16 (see chapter 4), despite the much higher virus dose used to infect mice with TKDM21. Two factors lead to the conclusion that a large proportion of SC16 genomes that persist in the PNS are products of viral DNA amplification during the acute phase. First, the major difference between TKDM21 and SC16 is the inability of TKDM21 to replicate in neurons. Second, the estimate of 22 viral DNA copies/LAT$^+$ neuron in TKDM21 infected ganglia was similar to the numbers detected in SC16 infected ganglia at spinal segments not innervating the inoculation site, ie. sites at which SC16 establishes latency without detectable replication (see chapter 4). Taken together, these data strongly indicate that viral DNA can persist in the PNS either before or after amplification in neurons.

The possibility that neurons may be able survive viral replication conflicts with the cytopathic effect caused by HSV in cell cultures. If neurons (which do not multiply) were destroyed, then it might be expected that individuals suffering repeated recurrent episodes would eventually experience sensory loss. However, loss of sensation is not associated with recurrent HSV infection (Roizman, 1965; Sabin, 1975). More recently, Simmons and Tscharke (1992)

used quantitative immunohistochemical techniques to analyse sensory neurons of BALB/c mice acutely infected with virulent HSV-1 strain SC16. They showed that the majority of these neurons could survive advanced stages of viral replication (up to at least γ gene expression). These cells would therefore have had the capacity to support viral DNA replication without destruction. This report supports the conclusions made in this and the previous chapter. It is noted, however, that Margolis *et al* (1992) were not able to demonstate persistence of replicated viral DNA in the PNS of mice following inoculation of HSV-1 strain KOS(M) directly into the sciatic nerve. However, under these conditions, the very high number of 'input' genomes/LAT^+ neuron (up to 140) may have masked products of viral DNA replication.

TKDM21 DNA recovered from latently infected mice lacked free genomic termini superficially resembling the structure of wild-type latent DNA. Most likely, the endless nature of latent TKDM21 DNA represents a circular episomal structure, corresponding to the proposed configuration of initial templates for DNA replication in infected cells (Poffenberger and Roizman, 1985; Garber *et al*, 1993). However, it is possible that this DNA may be integrated into host genetic material. In this respect, two previous reports have indicated that at least a proportion of viral DNA recovered from tissue latently infected with replication competent virus may be integrated into host cell DNA (Puga *et al*, 1984; Mellerick & Fraser, 1987). If TKDM21 genomes integrated via the repeated regions at specific sites in cellular DNA, then novel bands

corresponding to the virus-host junction may have been expected to be observed. However, the sizes of virus-specific bands detected in TKDM21 infected ganglionic DNA probed with pBKSP1 and pSB-6 were indistinguishable from those detected in samples containing HSV-1 strain SC16 DNA (ie. reconstructions, acutely infected Vero cells and infected ganglia). Therefore, if integration did occur at specific sites in cellular DNA, it was unlikely to have been via sequences within the junction or *Bam*HI-B regions of the viral genome. However, integration via the repeat regions of the viral genome, at random or multiple sites cannot be excluded. If this did occur, the junction region would remain intact, and novel fragments corresponding to virus-host junction would not be observed.

Cytoplasmic LATs have not previously been described in latently infected ganglia, and a protein product has not been identified *in vivo*. In some experiments in the present study strong cytoplasmic staining with LAT probe pSLAT-4 was observed in a small proportion (<1%) of LAT^+ neurons. Detection of LAT-specific cytoplasmic staining has several potential explanations. First, this may be the first detection *in vivo* of latency associated mRNAs, that might give rise, for instance, to latency related proteins such as those reported by Doerig *et al* (1991a) *in vitro*. Failure to detect cytoplasmic mRNAs by *in situ* hybridization protocols is not uncommon. For example, EBV RNAs encoding viral nuclear antigens are readily detectable within neuronal nuclei during latency but cannot be detected within the cytoplasm of

these cells, suggesting a lack of sensitivity (Lawrence *et al.*, 1989; Xing and Lawrence, 1991). Second, cytoplasmic LATs may be a feature of neurons undergoing spontaneous reactivation; ie. the staining represents viral DNA or LATs associated with productive infection. This is unlikely given that spontaneous reactivation has never been detected in the C57BL10 mouse latency model described here (A Simmons, personal communication and this thesis). Further, cytoplasmic staining was observed in neurons of mice infected with the TK deficient mutant TKDM21, which has been extensively characterized and cannot reactivate in the absence of complementary wild-type virus (Efstathiou *et al.*, 1989). Also, despite an intensive search, viral antigens could not be detected in the material in which cytoplasmic LATs were identified. A third possibility is that cytoplasmic staining is an artifact caused by leakage of LATs from the nucleus prior to the *in situ* hybridization procedure. Although this cannot be excluded, it is thought to be very unlikely due to the efficient nucleic acid fixing ability of PLP, and the characteristic high levels of LAT staining confined to nuclei in neighbouring neurons.

6. DISCUSSION

This chapter examines the differences and similarities between HSV latency and the latent infections established by two other human herpesviruses, namely EBV and VZV. Particular reference is made to the configuration and transcriptional activity of latent viral DNA. Knowledge about the molecular nature of the remaining human herpesviruses (ie. CMV, HHV-6 and HHV-7) was considered too rudimentary for inclusion here.

The latent genome of EBV has been studied intensively. EBV is a gammaherpesvirus that is carried asymptomatically by more than 95% of adults (Miller, 1990). Latent infection is established in circulating B lymphocytes and the molecular characteristics of latency have been studied in human B lymphocyte cell lines eg. Raji cells, derived from a Burkitt lymphoma biopsy (Pulvertaft, 1965). Permissive infection develops in a small subset of latently infected B lymphocytes, resulting in the generation of linear virion DNA (Rocchi *et al.*, 1977). Latently infected (ie. non-producer) dividing B lymphocytes characteristically contain multiple copies of the EBV genome per cell (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1971, 1972; Nonoyama *et al.*, 1973; Kawai *et al.*, 1973; Lindahl *et al.*, 1976; Ernberg *et al.*, 1977), which are a result of viral DNA amplification (Sudgen *et al.*, 1979). However, like HSV, viral DNA amplification is not a prerequisite for the establishment of latency (Hurley and Thorley-Lawson, 1988). Latent EBV genomes are

episomal molecules (Adams and Lindahl, 1975; Lindahl *et al.*, 1976), in which the repeat sequences, which flank the DNA, are covalently linked to form circles (Dambaugh *et al.*, 1980). In cell culture the number of EBV copies per cell (approx. 50) remains constant over time, suggesting that replication of the latent genome is tightly linked to cell division (Stevens, 1989). Indeed, EBV episomes are replicated once per cell cycle by host cell DNA polymerase (Hampar *et al.*, 1974; Shaw, 1985; Adams, 1987) and replication follows circularization of input DNA (Gussander and Adams, 1984). In addition to persistence in an episomal form, EBV DNA can integrate into chromosomal DNA (Henderson *et al.*, 1983; Matsuo *et al.*, 1984, Lawrence *et al.,* 1988). Integrated EBV DNA has been detected in at least two cell lines. In one (an EBV-infected human Burkitts tumour cell line), there is a single copy of viral DNA per cell, which integrates through the EBV terminal repeat (TR) region on chromosome 1 at IP35, and results in deletion of about 15 kb of host cell DNA (Henderson *et al.*, 1983; Matsuo *et al.*, 1984). Integration via the TR region favours the hypothesis that integration occurs prior to circularization. In another cell line (latently infected fetal human lymphoblastoid cells) there is a single integrated genome at chromosome 4q25 (Henderson *et al.*, 1983; Matsuo *et al.*, 1984). In this instance, integration is thought to occur after initial circularization because (i) of the concurrent presence of episomes and (ii) the left and right ends of the viral genome are linked. Clearly, the physical characteristics of viral DNA in these two cell lines are very different, and integration is not chromosome-site-specific. The biological significance of

integrated molecules is not known, but episomal DNA is likely to be necessary for lytic-cycle EBV DNA replication because (i) circular EBV DNA copy number increases during the productive infection (Shaw, 1985) and (ii) EBV DNA replication follows circularization (Gussander and Adams, 1984).

In contrast to HSV infected neurons, cells harbouring latent EBV genomes express one or more virally encoded proteins. These include six nuclear antigens (designated EBNA-1, -2, -3a, -3b, -3c, and -LP) and three membrane proteins (designated LMP-1, -2a, and -2b) (Kieff and Liebowitz, 1990). Rowe *et al* (1992) have identified three forms of EBV latency. In the first form, only EBNA-1 is expressed; in the second all 6 EBNAs and all 3 LMPs are expressed; and in the third, EBNA-1 and the 3 LMPs are expressed in the absence of the other EBNAs. There is, therefore, some similarity between EBV and HSV in that both viruses have more than one pattern of persistence.

The EBV genome is progressively and extensively methylated in latently infected cells (Kinter and Sugden, 1981; Perlmann *et al.*, 1982; Diala and Koffman, 1983; Saemundsen *et al.*, 1983), and drugs that reduce DNA methylation increase the frequency of spontaneous activation to lytic infection (Saemundsen *et al.*, 1980). It is interesting to note that although latent HSV DNA (at least in the CNS) is not extensively methylated (Dressler *et al.*, 1987), treatment of ganglionic and CNS tissue from latently infected animals with demethylating agents does, like EBV, enhance reactivation

(Stephanopoulos *et al.*, 1988; Bernstein and Kappes, 1988; Whitby *et al.*, 1988). Further, EBV resembles HSV in that the latent genomes of both viruses are associated with nucleosomes or other DNA-binding proteins which may regulate gene expression (Shaw *et al.*, 1979).

VZV is, like HSV, an alphaherpesvirus. Latent VZV infection is poorly understood, mainly because there is no satisfactory experimental model. Nonetheless, several lines of evidence suggest that following primary infection in the skin and mucous membranes, the virus establishes latency in sensory ganglia: (i) Explantation and *in vitro* cultivation of such ganglia is followed by the appearance of VZV gene products (Vafai *et al.*, 1988). (ii) VZV nucleic acid sequences have been detected in human sensory ganglia by Southern blot and *in situ* hybridization (Hyman *et al.*, 1983; Gilden *et al.*, 1983, 1987; Croen *et al.*, 1988). (iii) Recrudescence (herpes zoster) is tightly confined to a single dermatome (Gelb, 1990).

Hyman *et al* (1983) detected VZV RNA in trigeminal ganglia of latently infected humans and estimated by *in situ* hybridization that up to 0.3% of neurons contained VZV RNA transcripts. Gilden *et al* (1983), using Southern blot hybridization, showed that latently infected human trigeminal ganglia contain between 0.28 and 1 copy of VZV DNA per cell. By assuming that 10% of ganglionic cells are neurons (Walz *et al.*, 1986), and that all VZV DNA resides in neurons and is transcriptionally active, it follows that there are

approximately 930-3300 VZV genome copies per latently infected cell. It is pertinent to note that the results presented in this thesis suggest that a large number of latent HSV genomes (ie. progeny genomes) are transcriptionally repressed and may possibly not reside in LAT^+ neurons (or neurons at all). Further, there is considerable controversy regarding the cell type harbouring latent VZV DNA. *In situ* hybridization studies of latently infected human trigeminal ganglia done by Hyman *et al* (1983) and Gilden *et al* (1987) both detected VZV RNA in sensory neurons. In contrast, a detailed study of VZV latency by Croen *et al* (1988) suggested that VZV genomes reside in the satellite cells which surround sensory neurons. Using *in situ* hybridization they detected VZV RNA transcripts in approximately 0.01-0.15% of satellite cells, whereas in the same ganglionic tissue HSV-1 LATs were detected exclusively in neuronal cells.

The physical state of latent VZV genomes and the molecular basis for reactivation are presently unknown. In order to maintain latent infection in satellite cells, the viral DNA may be in an integrated or circular episomal form, and, like EBV, replicate with the dividing cell population. The different clinical features of VZV and HSV reactivation (ie. lesions are more extensive in zoster and appear much less frequently than herpes simplex) indicate that there are significant differences in either the nature of the latent state or the process of reactivation. It has been suggested by Croen *et al* (1988) that reactivation of VZV in satellite cells might result in spread of productive

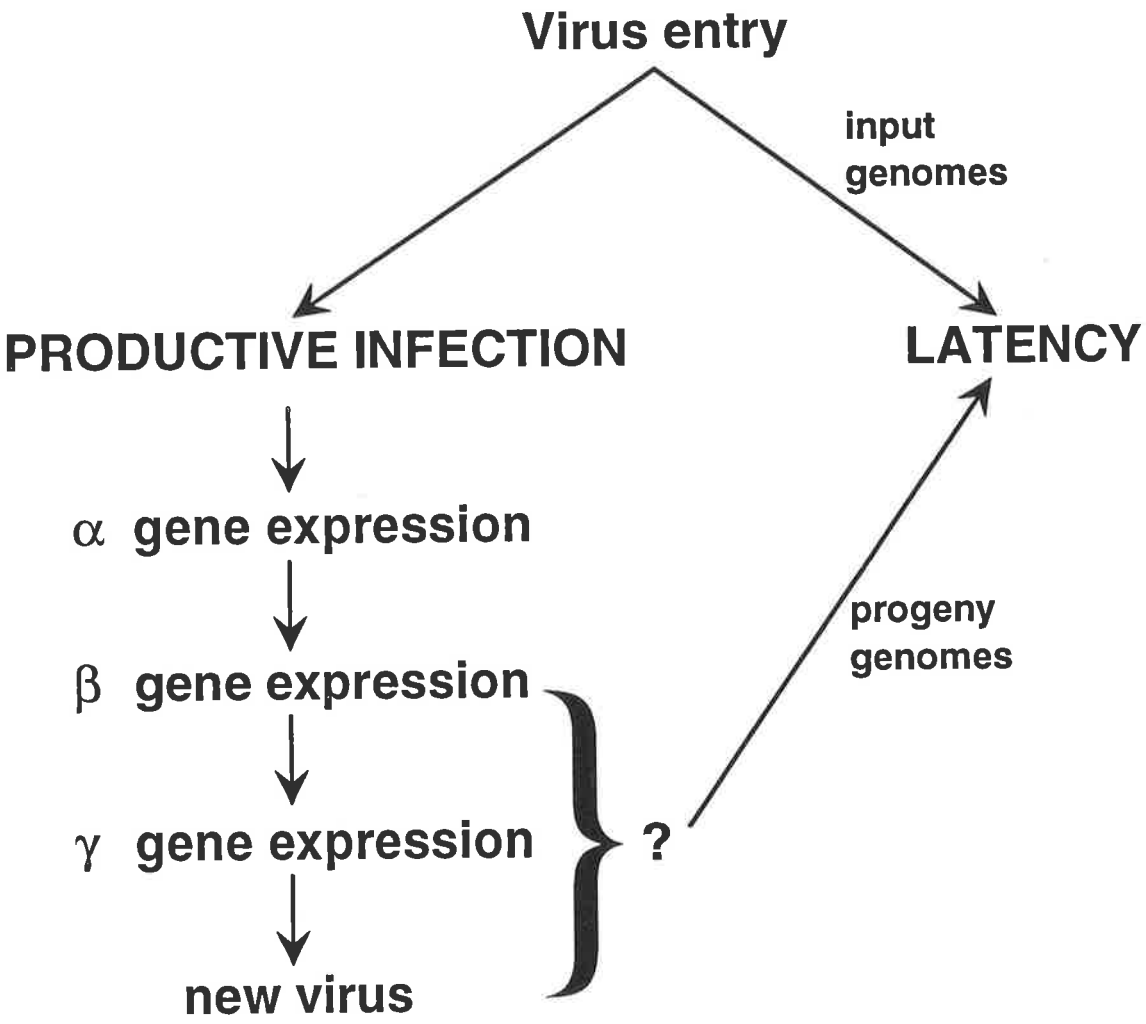
infection to many adjacent neurons, causing anterograde spread of virus to large areas of the dermatome.

A better understanding of HSV latency is dependent on elucidation of the molecular events that occur during the establishment of latent infection. This thesis presents evidence suggesting that the pathways leading to productive and latent infection can diverge both before and after viral DNA amplification, leading to persistence of two populations of latent genomes (Figure 6.1), distinguished by different transcriptional activities, and having potentially different biological significance. Although HSV is in some respects unique it is possible that the study of other herpesviruses may provide useful insight into the mechanisms by which HSV persists in the nervous system. Finally, it is clear that a more detailed molecular study of HSV persistence will be assisted by the development of an *in vitro* model that effectively mimics neuronal latency.

Figure 6.1

Schematic diagram of the HSV replicative cycle depicting the two proposed molecular pathways leading to the establishment of HSV latency. (i) Following virus entry latency can be established prior to the initiation of the replicative cycle, resulting in the persistence of input (ie. unamplified) viral genomes. (ii) Latency can be established at some stage after viral DNA replication (ie. after β gene expression), resulting in the persistence of progeny (ie. amplified) viral genomes.

Figure 6.1


Virus entry
input genomes
PRODUCTIVE INFECTION
LATENCY
α gene expression
β gene expression
γ gene expression
new virus
?
progeny genomes

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