ANALYSIS OF ESTABLISHMENT OF LATENT INFECTION WITH A VIRULENT STRAIN OF HERPES SIMPLEX VIRUS TYPE 1

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) mutants that have been denied the possibility of initiating infection by deletion of an essential gene retain the ability to persist in the host, from which it has been concluded that the pathways of latent and productive infection are divergent from a very early stage. A corollary of this conclusion, taking into account the belief that replication of the HSV genome is dependent on several virally encoded proteins, is that viral DNA cannot be amplified during the establishment of latency. This corollary is not supported by indirect assessment of latent DNA copy number following infection with replication competent viruses. Consequently, deletion mutants may not accurately represent the behaviour of replication-competent viruses, and the aim of this study was to determine whether a virulent strain of HSV-1 (SC16) that is not compromised in its ability to enter the lytic pathway by a defect in its genome, establishes latent infection without accompanying viral gene expression. Two strategies were devised to address this issue.

1. Viral gene expression (judged by presence of HSV antigens and mRNA) was detected in thoracic ganglia from three to seven days after inoculation of flank skin with HSV-1, strain SC16. Neurons expressing viral genes were confined to ganglia directly innervating inoculated skin (8th to 12th thoracic spinal segments). In contrast, latent infection was more widely distributed in the peripheral nervous system (6th thoracic to 1st lumbar segments), assessed by detection of latency associated RNA transcripts *in situ* and reactivation of infection *in vitro*.

2. In a different approach, dual-label technology (*in situ* hybridization for detection of LATs combined with immunohistochemical detection of viral proteins) was used to demonstrate that antigen-positive and latently infected neurons appear synchronously in spinal ganglia innervating inoculated skin during the earliest stages of productive ganglionic infection.

These results demonstrate that latent infection can be established without detectable expression of any genes associated with viral replication, implying that with replication-competent HSV-1, molecular pathways leading to productive and latent infection can diverge at a very early stage.

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

PETER GERALD SPECK

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PUBLICATIONS ARISING

Publications:

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Distribution of HSV antigen and LATs in ganglionic neurons during transition from acute to latent infection. P Speck, A Simmons. Poster presented at 14th International Herpesvirus Workshop, Nyborg, Denmark, August 1989.

Antigen expression need not precede latent HSV infection. P Speck, A Simmons. Poster presented at 15th International Herpesvirus Workshop, Washington DC, August 1990.

Antigen expression need not precede latent HSV infection. P Speck, A Simmons. Oral presentation at 8th International Congress of Virology, Berlin, August 1990.

Preservation of HSV infected neurons by a novel immunological mechanism mediated by $CD8^+$ lymphocytes. A Simmons, D Tscharke, P Speck. Poster presented at 16th International Herpesvirus Workshop, Pacific Grove, California, July 1991.

ABBREVIATIONS

A	adenine
AR	analytical reagent
ATP	adenosine triphosphate
C	cytosine
CTP	cytidine triphosphate
d	day
Da	Dalton
DDW	double distilled water
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
g	gravity
G	guanine
GTP	guanosine triphosphate
h	hour
ISH	*in situ* hybridization
kb	kilobase
kV	kilovolt
mA	milliamp
mm	millimeter
mRNA	messenger RNA
nm	nanometer
PFU	plaque forming unit
RNA	ribonucleic acid
RNAse	ribonuclease
RT	room temperature
SD	standard deviation
ss	single stranded
T	thymine
TCA	trichloroacetic acid
tRNA	transfer RNA
UTP	uridine triphosphate

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 The family herpesviridae

There are approximately 100 known herpesviruses of which six (herpes simplex virus types 1 and 2, varicella zoster virus, Epstein Barr virus, cytomegalovirus, and human herpesvirus 6) have been isolated from humans.

1.1.1 Definition of a herpesvirus

All herpesviruses possess the following common features of virion structure: (a) a genome of double stranded DNA; (b) an icosadeltahedral capsid approximately 100 nm in diameter and made up of 162 capsomeres; (c) an amorphous material, called tegument, surrounding the capsid and (d) an envelope with viral glycoprotein spikes on its surface (Roizman and Sears, 1990). Herpesvirions are indistinguishable from one another by electron microscopy (Watson, 1973).

1.1.2 Biological properties

All herpesviruses share a number of characteristics: they specify a large number of the enzymes involved in nucleic acid metabolism and DNA synthesis, replication of viral DNA and assembly of capsids both occur in the nucleus and all herpesviruses appear to be capable of establishing a lifelong latent infection in their host, (Roizman, 1990).

Herpesviruses are divided into three sub-families, the alpha-, beta-, and gamma-herpesviruses, on the basis of host range and tissue tropism *in vivo*, by host range, growth rate and cytopathology in cell culture (Honess and Watson, 1977; Roizman *et al*, 1981).

Alphaherpesviruses. There are three human alphaherpesviruses: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV). Viruses of this group are distinguished by having a broad host range in experimental animals, and by rapid spread and a relatively short reproductive cycle ($\leq$ 18 hours) in culture. Alphaherpesviruses generally destroy productively infected cells (Honess, 1984).

Betaherpesviruses, also known as cytomegaloviruses because of the enlargement ("cytomegaly") frequently seen in infected cells, include human cytomegalovirus and murine cytomegalovirus. Viruses of this group are typically species-specific *in vivo* and *in vitro* (Wright, 1973) and grow slowly in cultured cells. Infected cells may contain non-infectious particles ("dense" bodies: Craighead *et al*, 1972) comprised of virus tegument and envelope polypeptides. The biologically relevant site of persistence of human cytomegalovirus is unclear, but virus can be isolated from salivary glands, lymphoreticular cells and kidneys. Persistent cytomegalovirus infection is usually asymptomatic but may produce disseminated infection and serious disease in neonates or in immunocompromised adults of their natural hosts (Honess, 1984).

Gammaherpesviruses, which are generally lymphotropic, are divided into two subgroups, γ_1 and γ_2, on the basis of tropism for either B or T lymphocytes respectively (Deinhardt *et al*, 1974; Frank *et al*, 1976) and on the basis of genetic complexity and % guanine and cytosine content (Honess, 1984). Epstein-Barr virus (EBV), the causative agent of human infectious mononucleosis, is a γ_1 herpesvirus that causes immortalization of B-lymphocytes *in vitro*. EBV genomes are maintained as circular episomes with restricted virus expression (Kieff and Leibowitz, 1990). γ_2 viruses (e.g. herpesvirus saimiri) can be isolated from T-lymphocytes of the majority of adult members of their natural host species and in related species some viruses of this subgroup can cause lymphoproliferative disease (Honess, 1984).

1.2 Herpes simplex virus

HSV is one of the most intensively investigated vertebrate viruses. The ability of HSV to establish, maintain, and reactivate from latent infection contributes greatly to the impact of the virus on humans but despite decades of research latency remains, in molecular terms, a poorly understood phenomenon.

1.2.1 History

The earliest descriptions of herpes simplex date back to ancient Greek times and the word herpes is derived from the Greek word "Ερπης", meaning creeping thing (Beswick, 1962). Around 484 BC Hippocrates used the term "herpes" to describe spreading cutaneous lesions. More than 2000 years later it was shown that herpes

simplex could be transmitted from one human being to another (Vidal, 1873) and in 1911 HSV was successfully transferred to rabbits by Gruter in what was probably the first application of an animal model to study of herpesviruses (Gruter, 1920). In 1921 Luger and Lauda showed HSV to be filterable. At that time the nature of viruses was poorly understood and controversy arose regarding the ability of herpes simplex to recur. Doerr (1938) proposed that herpes simplex was an endogenous production of a virus-like organism under the influence of unknown physiological stimuli. A landmark paper by Burnet and Williams (1939) suggested that primary lesions, often in childhood, lead to lifelong persistence of virus in ganglia and all subsequent research supports their proposal.

1.2.2 Components of the infectious agent of herpes simplex

The core of the mature virion contains a double stranded DNA genome consisting of about 150,000 base pairs with a guanine/cytosine content of 67 to 69% (Russell and Crawford, 1964; Becker *et al*, 1968; Kieff *et al*, 1971). The complete genome of HSV type 1 (strain 17) has been sequenced and encodes at least 72 polypeptides, the function of many remaining unknown (Murchie and McGeoch, 1982; McGeogh *et al*, 1985, 1986a, 1986b, 1988a; McGeoch and Davison, 1986; Perry *et al*, 1986; Perry and McGeoch, 1988). The genome can be divided into long and short components, designated L and S respectively, each containing a central unique region flanked by inverted repeats (Sheldrick and Berthelot, 1974; Wadsworth *et al*, 1975) (see Fig 1.4). The L and S components can invert in relation to each other leading to the existence of 4 isomers (Hayward *et al*, 1975;

Delius and Clements, 1976), which are found in equimolar proportions in viral DNA extracted from infected cells (Clements *et al*, 1976; Jacob *et al*, 1979).

Two types of herpes simplex virus, designated 1 and 2, can be distinguished serologically (Schneweis, 1962). Type 1 is usually associated with peri-oral lesions while type 2 is generally associated with genital lesions (Dowdle *et al*, 1967). DNAs of types 1 and 2 are colinear with approximately 47% matching of base pairs (Kieff *et al*, 1972).

HSV virions contain at least 33 virally encoded polypeptides (Spear and Roizman, 1972; Heine *et al*, 1974), but no host protein has been detected in purified virions (Roizman and Sears, 1990). At least nine glycosylated polypeptides (designated gB, gC, gD, gE, gG, gH, gI, gJ, gL) are located on the surface of the virion and are thought to mediate infectivity (Ackerman *et al*, 1986; Gompels and Minson, 1986; Richman *et al*, 1986; Longnecker *et al*, 1987; Johnson *et al*, 1988; Bell *et al*, 1990; Hutchinson *et al*, 1992).

The tegument of each virion contains 500-1000 copies (Heine *et al*, 1974) of a protein of ~65,000 kD molecular weight, variously known as α transinducing factor (αTIF), infected cell polypeptide (ICP)25, virion protein (VP)16, or Vmw65, which interacts both with the viral genome and cellular transcription factors. The resulting complex, called α-transinducing complex, stimulates transcription of viral genes (Post *et al*, 1981; Batterson and Roizman, 1983;

Campbell *et al*, 1984; Pellett *et al*, 1985; Marsden *et al*, 1987; McKnight *et al*, 1987; Gerster and Roeder, 1988; O'Hare *et al*, 1988; Preston *et al*, 1988). The tegument also contains the virion host shut off protein (VHS) that destabilizes mRNA molecules (Nishioka and Silverstein, 1977 and 1978; Fenwick and Walker, 1978; Read and Frenkel, 1983; Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987; Kwong *et al*, 1988). The VHS protein shuts down host cell protein synthesis and speeds transition between the different classes of viral genes.

1.2.3 Replication of HSV

Replication of the virus can be considered in terms of (a) attachment to the cell (b) entry into the cell (c) transport to the nucleus (d) transcription of viral genes (e) replication of viral DNA (f) synthesis of viral structural proteins (g) assembly of new capsids (h) budding of capsids from the nucleus and (i) assembly of infectious, enveloped, virions (reviewed in Roizman and Sears, 1990).

Attachment of herpes simplex virions to cells is mediated, at least in part, by the binding of virions to heparan sulphate moieties of cell surface proteoglycans (Wudunn and Spear, 1989). Penetration requires three virion glycoproteins (gB, gD and gH) (Sarmiento *et al*, 1979; Little *et al*, 1981; Cai *et al*, 1987, 1988; Fuller and Spear, 1987; Highlander *et al*, 1987, 1988; Campadelli-Fiume *et al*, 1988; Ligas and Johnson, 1988; Fuller *et al*, 1989), is enhanced by the presence of glycoprotein C (Herold *et al*, 1991), and occurs by the fusion of the virion envelope with the cell plasma membrane (Huang and Wagner, 1964; Manservigi

et al, 1977; Johnson *et al*, 1984). After the virion penetrates the cell membrane, the uncoated capsid and tegument proteins are transported to nuclear pores and viral DNA is released into the nucleus (Batterson *et al*, 1983), where transcription of viral genes occurs. HSV gene expression is tightly regulated and viral genes are divided into three somewhat overlapping temporal classes, designated as α, β and γ, or immediate-early, early, and late (IE, E, L) (Honess and Roizman, 1973, 1974 and 1975; Jones and Roizman, 1979). Although herpesvirus genes carry transcriptional and translational motifs similar to those of other DNA viruses that infect higher eukaryotic cells, mRNAs arising from most genes are not spliced (Roizman and Sears, 1990). Viral DNA is thought to be transcribed throughout the reproductive cycle by host RNA polymerase II (Costanzo *et al*, 1977).

The α genes, designated 0, 4, 22, 27, and 47 are by definition expressed in the absence of *de novo* viral protein synthesis and a feature of these genes is the presence of an upstream cis-acting element (Mackem and Roizman, 1982a, 1982b) containing the essential motif TAATGARAT (where R is a purine residue) in their 5' regulatory regions (Cordingley *et al*, 1983; Campbell *et al*, 1984; Lang *et al*, 1984; Preston *et al*, 1984). Synthesis of α polypeptides peaks 2 to 4 hours after infection in cell culture but α proteins continue to accumulate until late in infection (Honess and Roizman, 1974). All α proteins appear to have regulatory functions and are likely to be required for synthesis of subsequent polypeptide groups but precise functions of most α genes are not known. α4 and α27 are

essential for viral replication (Preston, 1979; Dixon and Schaffer, 1980; Deluca *et al*, 1985; Sacks *et al*, 1985; Smith and Schaffer, 1987), while α22 and α47 are dispensable in some cell culture systems (Post and Roizman, 1981; Mavromara-Nazos *et al*, 1986). α0 promiscuously stimulates transfected genes in transient expression systems but is not an essential gene for viral replication in cultured cells (Everett, 1984, 1986; O'Hare and Hayward, 1984, 1985; Gelman and Silverstein, 1985; Sacks and Schaffer, 1987).

In co-ordinately infected cultured cells, β gene products peak 5-7 hours after infection (Honess and Roizman, 1974). Most virally encoded proteins involved in nucleic acid metabolism are products of β genes (reviewed in Knipe, 1989), including viral thymidine kinase and ICP8, a DNA binding protein. The latter is involved in organizing DNA replicative enzymes (Conley *et al*, 1981; Littler *et al*, 1983). Viral thymidine kinase (TK), one of the best characterised proteins of HSV, phosphorylates a wide range of nucleoside analogues that are not phosphorylated efficiently by cellular kinases (Kit and Dubbs, 1963, 1965; Klemperer *et al*, 1967). HSV TK expression is dispensable for infection of dividing cells in culture but is essential for infection of non-dividing cells (Jamieson and Subak-Sharpe, 1974).

γ genes, which mainly encode viral structural proteins are subdivided into γ_1, and γ_2, with the latter requiring viral DNA synthesis for their expression (Holland *et al*, 1979, 1980; Conley *et al*, 1981; Costa *et al*, 1981). In co-ordinately infected

cultured cells, γ gene products are synthesized at increasing rates until at least 12 hours after infection (Honess and Roizman, 1974). VP16 is a γ_2 gene and despite strong expression late in the replicative cycle does not, for reasons that are unclear, at that time transactivate viral α genes (Roizman and Sears, 1990).

The mechanism by which HSV DNA replicates is unclear. In lysates of infected cells, viral DNA has been variously reported to be present in the form of branched molecules, circles, lariats and multi-unit length genomes (Ben-Porat *et al*, 1976; Hirsch *et al*, 1976, 1977; Jean and Ben-Porat, 1976; Schlomai *et al*, 1976; Friedman *et al*, 1977; Jacob and Roizman, 1977) and a rolling circle model of replication has been proposed (Jacob *et al*, 1979).

Capsids are assembled in the nucleus (Ben-Porat and Kaplan, 1973) and newly synthesized DNA is processed and packaged into preformed empty capsids (Deiss *et al*, 1986; Deiss and Frenkel, 1986). Late in infection, thick concave or convex patches become visible by electron microscopy on the nuclear membranes of infected cells and envelopment probably takes place at these patches. The time required for a complete replicative cycle of HSV in cultured cells is approximately 18-20 hours (Darlington and Granoff, 1973). It is widely accepted that, at least in culture systems, cells productively infected with HSV do not survive.

1.3 Pathogenesis of HSV infection

During primary infection, which typically involves skin or mucous membranes, HSV is taken up by nerve endings and travels to sensory neurons, in which latency, the hallmark of herpes simplex, is established.

1.3.1 Primary infection

Infection is transmitted by direct contact. Primary oral infection is usually asymptomatic, but when symptoms are evident the typical manifestation is gingivostomatitis. Herpetic gingivostomatitis is most prevalent in children less than 5 years old (Dodd *et al*, 1938; Whitley, 1990). Rarely, primary infection with HSV in infants causes zoster-like lesions involving a complete dermatome (Mok, 1971; Music *et al*, 1971). Primary genital infection, like oral infection, may be asymptomatic, but a substantial proportion (~50%) of cases are associated with painful blistering of genital skin and/or mucous membranes. (Nahmias *et al*, 1969; Corey and Spear, 1986).

During primary infection, viral replication at the site of inoculation is accompanied by necrosis and infiltration of inflammatory cells. Infected epidermal cells undergo characteristic changes, including fusion to form multinucleated giant cells. Condensation of nuclear chromatin is followed by nuclear degeneration and cell death (Darlington and Granoff, 1973). Vesicles containing large quantities of virus, cellular debris and inflammatory cells appear in the uppermost layers of the epidermis (Whitley, 1990). With healing, the

initially clear vesicular fluid becomes pustular and less virus is recoverable from it. Complete healing of primary lesions takes 2 to 3 weeks; scarring is uncommon (Buddingh *et al*, 1953).

1.3.2 Recrudescent and recurrent infection

HSV is characterized by its ability to cause recrudescent lesions, such as cold sores. Studies carried out on university students have shown that between 16% and 61% of subjects suffered one or more cold sores per year (Ship *et al*, 1960, 1961; Embil *et al*, 1975; Young *et al*, 1976; Friedman *et al*, 1977). The precise nature of the stimulus causing cold sores is not known, but they may be triggered by exposure to sunlight or wind, by fever, by menstruation, or by a severe emotional experience (Nahmias and Roizman, 1973). Like primary infections, recurrences may occur asymptomatically and in the case of HSV-1, surveys have found that 1-5% of individuals are excreting virus in the saliva at any moment in time (Buddingh *et al*, 1953; Stern *et al* 1959; Lindgren *et al* 1968; Hatherly *et al* 1980). Repeated sampling of patients with antibodies to herpes simplex virus type 1 have shown that 27% or more excreted virus in their saliva over study periods of several years (Cesario *et al*, 1969; Greenburgh *et al*, 1969; Douglas and Crouch 1970). Thus asymptomatic excretion of virus is common and may be an important mode of transmission.

Extensive epidemiological studies have shown that the incidence of genital herpes simplex infection resembles that of perioral infection. Viral shedding in the

absence of clinical signs is highly prevalent (Rattray *et al*, 1978; Adam *et al*, 1979; Ekwo *et al*, 1979; Corey, 1988). HSV type 2 recrudesces more frequently at genital sites than it does at perioral sites (Reeves *et al*, 1981) and conversely HSV type 1 recrudesces more frequently at perioral rather than genital sites. This may underlie the loose association of HSV-1 and HSV-2 with oral and genital regions respectively.

Recrudescent lesions, both perioral and genital, usually heal more quickly and are more localized than primary lesions. During the formation of a cold sore, a prodrome of pain, burning, tingling or itching, most commonly at the mucocutaneous junction of the lip, is followed within 24-48 hours by vesicles. These usually crust over in less than a week and healing is generally complete in 8-10 days (Spruance *et al*, 1977; Corey *et al*, 1983). For unknown reasons, recrudescent lesions are often located in a different part of the dermatome to the site of primary infection. For example primary oral infections are usually inside the mouth (gingivostomatitis) whilst recrudescent lesions are usually on the lip (cold sores). Similarly, primary genital infection in the female almost invariably involves the cervix whilst recrudescent lesions are usually vulval (Corey *et al*, 1983).

1.4 Clinical significance of herpes simplex

Herpetic lesions of skin and mucosa cause pain and suffering for millions of people worldwide and there are more serious consequences of infection for

neonates, for immunocompromised patients, and for patients infected in the eye (Nahmias and Roizman, 1973; Corey and Spear, 1986; Whitley, 1990). HSV infection acquired *in utero* or during birth can lead to blindness, mental retardation, or potentially lethal disseminated infection (Stagno and Whitley, 1985; Whitley and Hutto, 1985). Immunocompromised (e.g. AIDS) patients often suffer from locally aggressive herpetic lesions which take many weeks to heal (Siegal *et al*, 1981; Whitley, 1990). Ocular herpes simplex is one of the most common causes of unilateral blindness in the United States (Kaufman, 1978; Pavan-Langston, 1984). Unusual manifestations of herpes simplex include encephalitis, which can result from primary or recurrent infection and which usually causes devastating, often lethal, neurological damage (Olson *et al*, 1967; Corey and Spear, 1986). Disseminated herpes simplex in the immunocompetent adult is rare but, as its name implies, affects many organs in the body and is almost invariably lethal (Whittaker and Hardson, 1978; Whorton *et al*, 1983).

Serological studies have shown that HSV infection is ubiquitous: at age 15 over 90% of children in Brazil and in the United States have antibodies to herpes simplex virus in their blood (Black *et al*, 1974; Black, 1975). HSV type 2 specific serology has been used to estimate the prevalence of genital herpes simplex, which is strikingly common in adults. Estimates of individuals infected genitally range from 10 to 60 million in North America (Chuang *et al*, 1983; Guinan *et al*, 1985; Johnson *et al*, 1989; Whitley, 1990).

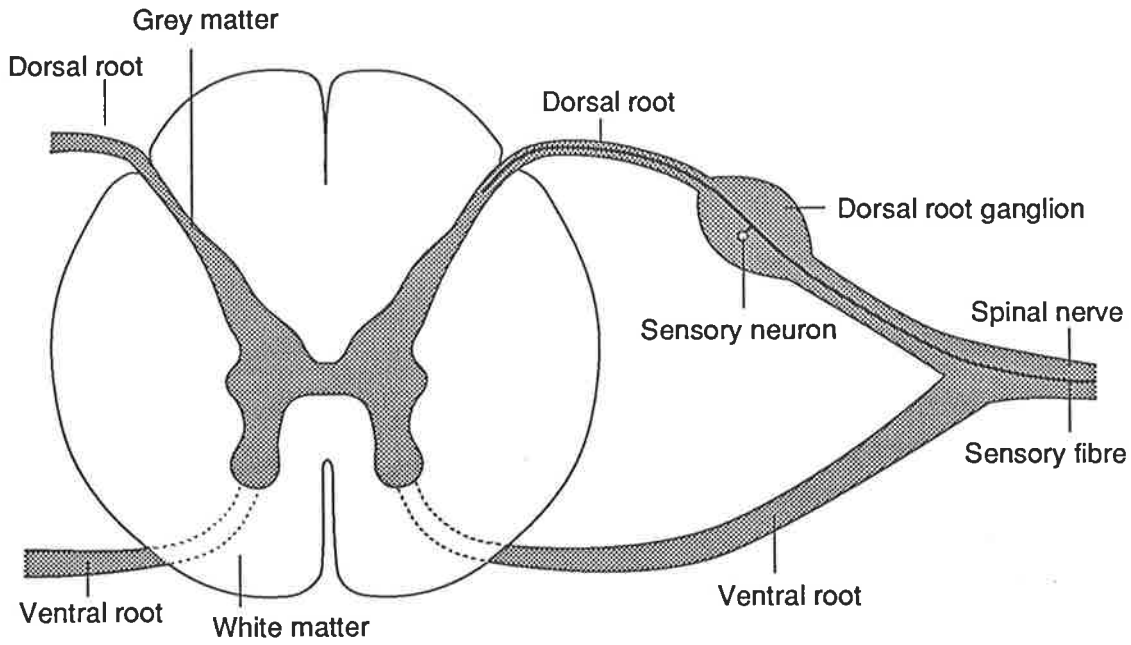
1.5 Latency

Latency is the hallmark of herpes simplex. In order to understand the pathogenesis of latent HSV infection, a basic knowledge of the anatomy of the peripheral nervous system is required.

1.5.1 The neurodermatome

The primitive plan upon which all vertebrates are built is bilaterally symmetrical and segmental and this pattern is reflected in the organisation of the nervous system, such that each segment of the cerebrospinal axis originates 2 dorsal sensory roots (Fig 1.1). The cell bodies of the primary sensory neurons are situated in dorsal root ganglia and the axons of these unipolar cells bifurcate at a T-junction to emit the central and peripheral processes of the sensory nerve fibre. Cell bodies of ganglionic neurons are large (up to ~70μ in diameter), rounded and have a large nucleus up to ~25μ in diameter (Fig 1.2). Each neuron is surrounded by a single layer of flattened cells called capsular cells (Warwick and Williams, 1973). Neurons occupy most of the volume but comprise only 1-10% of the number of cells in a ganglion (Pannese, 1964; Pannese *et al*, 1972, 1973; Walz *et al*, 1976). Each dorsal root ganglion innervates a segment of skin called a dermatome (Foerster, 1933; Warwick and Williams, 1973) (Fig 1.3) and successive dermatomes of the trunk overlap their neighbours by approximately 50% (Head, 1893, 1920; Sherrington, 1906; Foerster, 1933; Keegan, 1943). Thus, the ninth thoracic dermatome (designated T9) overlaps T8 rostrally and T10 caudally and T9 and T11 meet around the middle of T10. The functional unit of

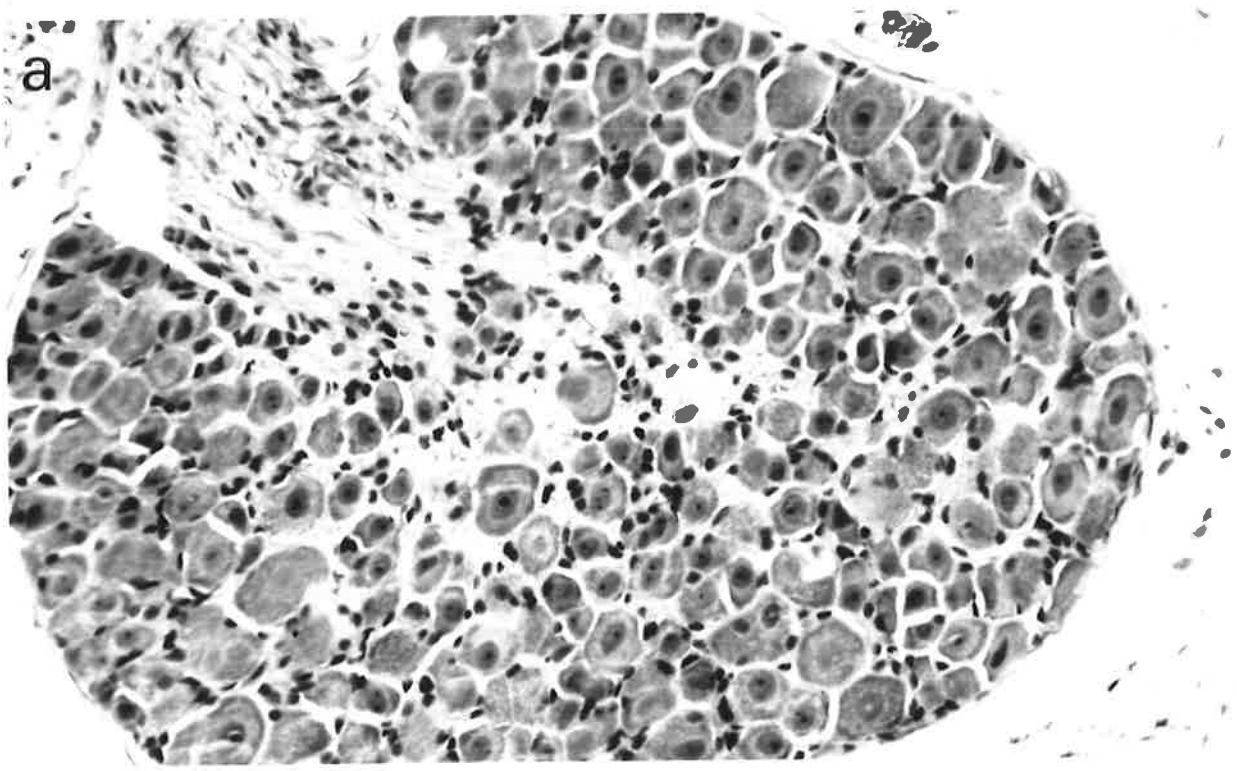
Figure 1.1. Schematic diagram showing formation of the spinal nerve from the dorsal and ventral roots of the spinal cord and location of a dorsal root ganglion, which contains cell bodies of primary sensory neurons.


Grey matter
Dorsal root
Dorsal root
Dorsal root ganglion
Sensory neuron
Spinal nerve
Sensory fibre
Ventral root
White matter
Ventral root

SPINAL CORD

Figure 1.2. (a) Photomicrograph (x160) of a section through a spinal ganglion of a mouse, showing neurons (large rounded cells), capsular cells (small flattened cells surrounding neurons) and nerve fibres (top left corner).

(b) Photomicrograph (x625) of a section through a spinal ganglion. Nucleus of a capsular cell is arrowed.


a

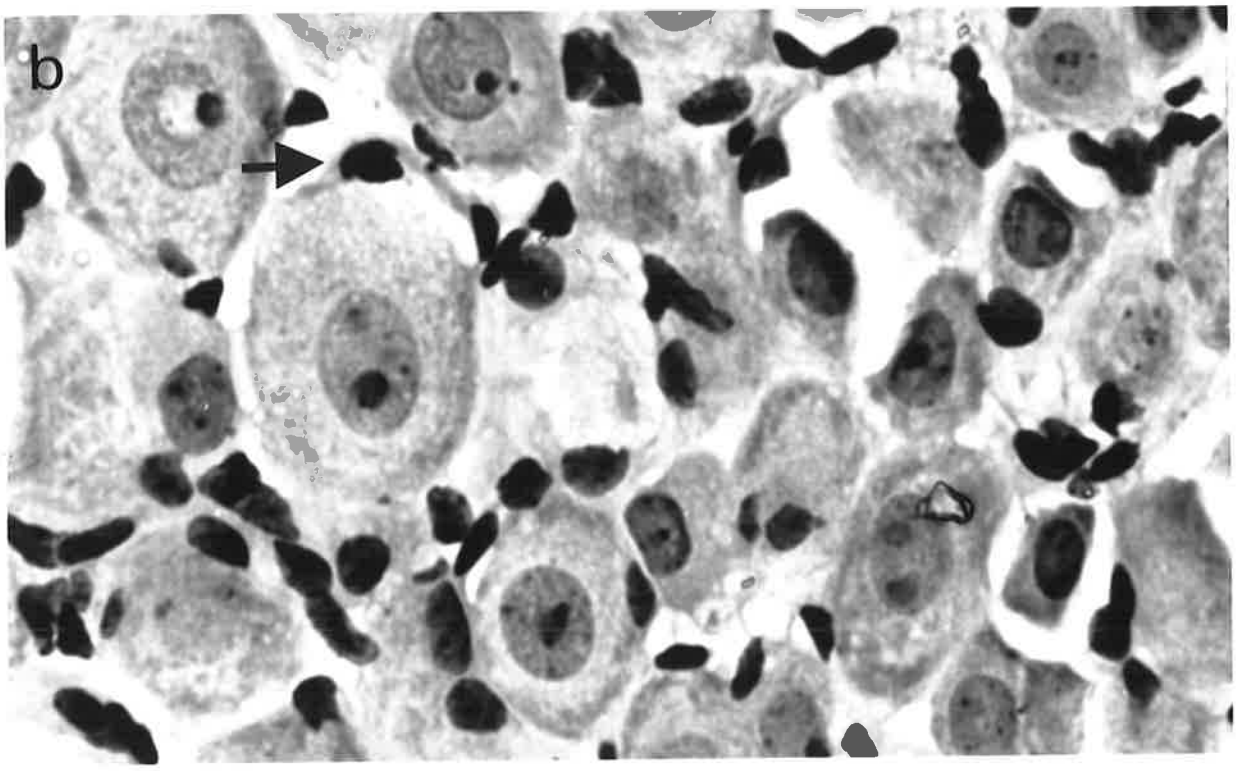
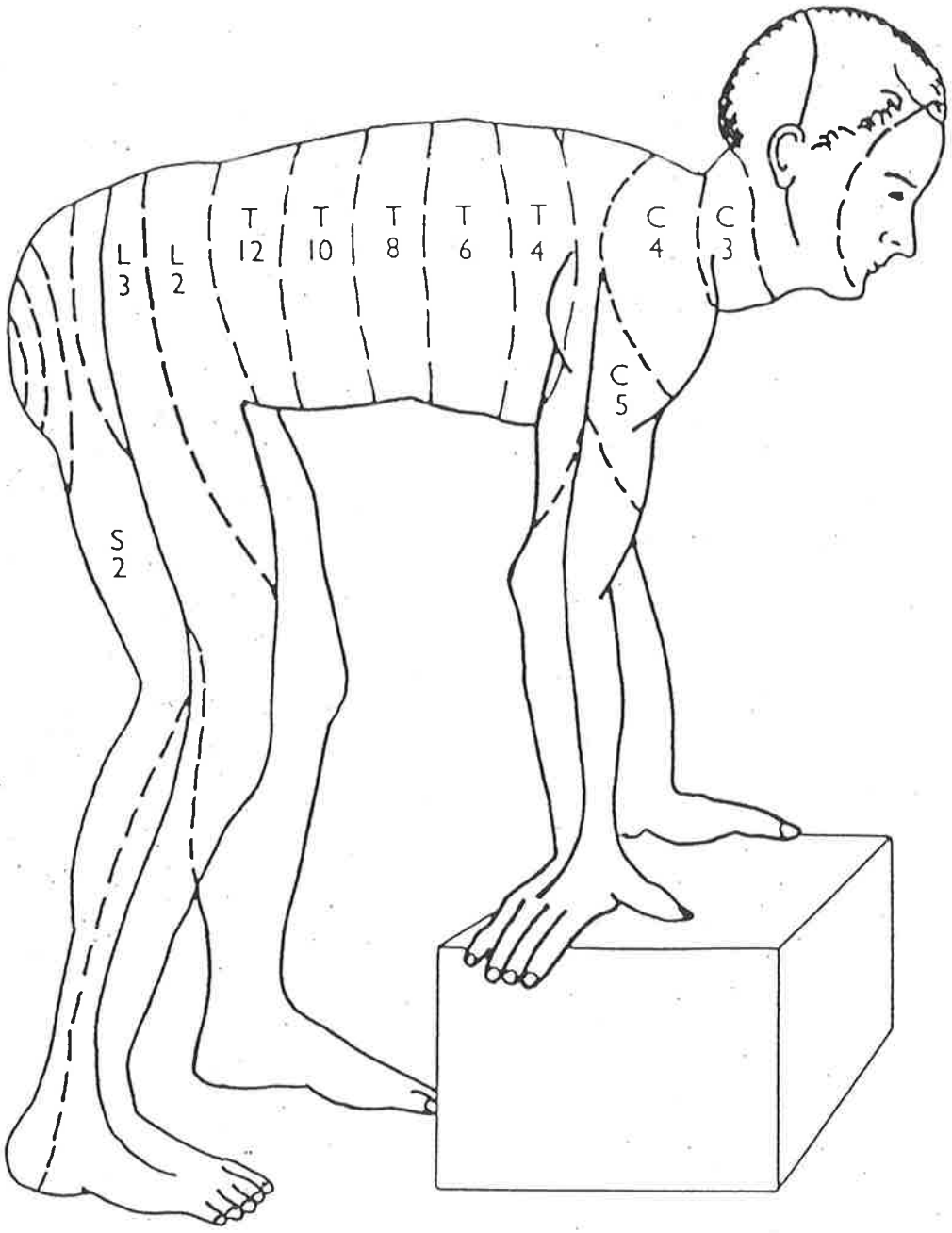

b

Figure 1.3. The segmental distribution of the nerves supplying human skin. Each dermatome is labelled according to the vertebral level at which the corresponding nerve leaves the spinal column. (C=cervical, T=thoracic, L=lumbar, S=sacral). Therefore, for example, the nerve supplying the dermatome marked T10 leaves the spine at the level of the tenth thoracic vertebra. There is considerable overlap in the innervation so, for instance, T9 (not marked) would cover the caudal half of T8 and the rostral half of T10. Mice have 13 thoracic segments, whereas humans have 12. Modified to the quadruped position from Gray's Anatomy, 35th edition, Longman, Edinburgh, by Dr A Simmons.


L 3
L 2
T 12
T 10
T 8
T 6
T 4
C 4
C 3
C 5
S 2

the nervous system comprising the nerve fibres, dorsal root ganglion and patch of skin is referred to as a neurodermatome and it is within this entity that primary infection, latency, and recrudescence are located.

1.5.2 Evidence for persistence of HSV in neural tissue

As early as 1904, Cushing noted a link between HSV and the nervous system and reported that surgical trauma to the trigeminal ganglion often gave rise to herpetic lesions on the face. Subsequent reports confirmed this observation (Carton and Kilbourne, 1952; reviewed in Baringer, 1975). Experimental zosteriform spread in animals further strengthened the link between the causative agent of herpes simplex and the neurodermatone (Teague and Goodpasture, 1923). Indeed animal models have been particularly useful for investigating the pathogenesis and immunology of herpes simplex, and to a certain extent for characterising latency at the molecular level. HSV can be reactivated from sensory ganglia of latently infected rabbits (Stevens *et al*, 1972), mice (Stevens and Cook, 1971) and guinea pigs (Scriba, 1975) by culturing ganglionic tissue *in vitro* for several days. Infectious virus has also been recovered from trigeminal ganglia of human cadavers following 10 to 45 days of explant culture (Bastian *et al*, 1972; Baringer and Swoveland, 1973).

Early experiments suggested that neurons may be required for persistence of HSV. For instance, Cook and Stevens (1976), studied the spectrum of tissues from which HSV could be reactivated *in vitro*, following recovery of mice from

systemic infection. Virus was detected in brain, spinal cord, adrenal medulla, and spinal ganglia all of which contain neurons, but not in non-neuronal sites such as bone marrow, lymph nodes, spleen, kidney, lung or liver. Subsequent experiments have confirmed that neurons play a vital role in latency. During reactivation, viral antigens, viral particles and thymidine incorporation into viral DNA are associated with neurons before other cell types (Cook *et al*, 1974). When replication of HSV during reactivation *in vitro* is precluded by a temperature-sensitive mutation in the viral genome, viral antigens are, at the non-permissive temperature, detected exclusively in neurons (McLennan and Darby, 1980). Furthermore viral transcription has been detected in sensory neurons during latency using *in situ* hybridization (Stroop *et al*, 1984; Stevens *et al*, 1987; see 1.5.8 *et sequelae*).

1.5.3 Evidence for persistence of HSV in peripheral tissues

Explant culture, which provides an operational definition of latency, suggests that HSV might persist in peripheral tissues, notably the eyes, ears and footpads, of experimentally infected animals. There are three possible explanations for recovery of HSV from the periphery during periods of latency: (i) Infectious virus may indeed persist in a non-infectious form, resembling neuronal latency. (ii) A very small amount of infectious virus may be present in skin at time of excision as a result of asymptomatic shedding following spontaneous reactivation in the PNS. Explant culture cannot distinguish between true latency and low level productive infection. This, the skin trigger hypothesis (Hill and Blyth, 1976),

states that spontaneous reactivation often occurs in neurons, leading to the frequent arrival of minute amounts of virus at the periphery, perhaps initiating tiny foci of productive infection. Certain situations, such as trauma or UV light enhance peripheral productive infection, leading to recrudescence. (iii) There might be low level persistence of infectious virus in skin.

Several observations suggest that detection of virus in peripheral tissues during latency may not be an artifact caused by seeding of the skin from the PNS: (i) Clements and Subak-Sharpe (1988) showed that neither treatment with the antiviral drug acyclovir nor sectioning of the femoral and sciatic nerves, nor both treatments combined, prevented recovery of virus from explanted mouse footpads. (ii) Scriba (1977) described peripheral latency in guinea pig footpads infected with either HSV-1 or HSV-2. Notably, HSV-2 could be reactivated *in vitro* preferentially from the footpad rather than the innervating sensory ganglia, and HSV-1, in a limited study (8 animals) was reactivated exclusively from the footpad. (iii) Ocular infection of mice and rabbits provides two additional systems in which peripheral latency has been detected by virological criteria (explant culture) (Openshaw, 1983; Claoue *et al*, 1990; Abghari and Stulting, 1988; O'Brien and Taylor, 1989; Cook *et al*, 1987). *In vitro* reactivation experiments localize putative latency to the retina (Openshaw, 1983) which contains neurons, and to the cornea and anterior uvea, which do not contain neuronal cell bodies. As in the mouse footpad model, acyclovir treatment of latently infected animals (Claoue *et al*, 1990) did not decrease the frequency of

virus isolation by explant culture.

Some recent studies have detected HSV nucleic acid sequences in herpetic corneas using *in situ* hybridization (Sabbaga *et al*, 1988) or polymerase chain reaction (PCR; Rong *et al*, 1991), in the absence of infectious virus in tears. Sabbaga *et al* (1988) detected HSV nucleic acid sequences in all murine corneal layers during latency. In contrast, during acute infection HSV nucleic acid sequences were confined principally to the corneal epithelium and a few stromal cells. Low stringency conditions used for hybridization and washing of sections greatly weakened this study. Rong *et al* (1991) studied human corneas using sets of PCR primers specific for two regions of the HSV genome (thymidine kinase and the LATs region). Whilst many biopsies appeared to contain HSV nucleic acid sequences, some were positive for only one or other of the target sequences studied. The authors speculated that incomplete HSV genomes might persist in the cornea. At the time of writing this thesis it remains controversial whether peripheral latency plays an important role in the biology of herpes simplex.

1.5.4 HSV transport in the nervous system

The mechanism by which HSV is translocated from skin to the peripheral nervous system has been extensively investigated. Evidence suggests that (1) virus travels along nerve trunks and not in blood or in the lymphatic system and (2) virus travels intra-axonally.

Wildy (1967) showed that blood- or lymph-borne virus does not play a significant role in infecting dorsal root ganglia by monitoring recovery of virus from lymph glands and nervous system following intradermal or intravenous inoculation. Viraemia after intravenous infection was found to last only a few hours and did not result in cerebral infection. In contrast, peripheral inoculation resulted in invasion of the central nervous system which was prevented by transection of the local peripheral nerve.

There is considerable evidence to support the view, first put by Goodpasture and Teague (1923), that virus travels intra-axonally. First, the rates of translocation of HSV and pseudorabies virus from skin to ganglia range from 2 through 10 mm/hr (Cook and Stevens 1973; McCracken *et al* 1973; Field and Hill 1974; Kristensson *et al* 1974), which is too fast to be consistent with sequential infection of Schwann cells (Wildy *et al*, 1982), but resembles the rate of retrograde axonal transport (Edstrom and Hanson, 1973; Ochs, 1974; Lubinska, 1975; Stoeckel *et al*, 1975; reviewed in Kristensson, 1978). Second, after introduction of HSV into one eye, virus spreads along previously mapped ocular neural pathways to reach specific foci (contralateral but not ipsilateral superior colliculus and lateral geniculate body) within the brain (Kristensson *et al*, 1974). Third, electron microscope studies have consistently shown virions within the axoplasm of peripheral neurons (Hill *et al*, 1972; Cook and Stevens, 1973; Baringer and Swoveland, 1974; Krisstensson *et al*, 1974) but support cells were found to contain morphologically incomplete virions (Cook and Stevens, 1973) or no

virions at all (Dillard *et al*, 1972).

1.5.5 Nature of the latent state

During latency infectious virus cannot be recovered and viral antigens, readily detected by immunohistochemical means in productively infected tissue, are not present in latently infected cells (reviewed in Wildy, 1982). Reports that latently infected tissue contains virally encoded proteins (Yamamoto *et al*, 1977; Green *et al*, 1981) have not been confirmed.

Studies with the antiviral drug acyclovir strongly suggest that viral DNA replication is not essential for maintenance of latency. Acyclovir is a guanosine analogue that is phosphorylated by viral thymidine kinase to a form which is incorporated into growing DNA chains by viral DNA polymerase. This terminates chain growth because acyclovir lacks the necessary 3' hydroxyl group to react with incoming nucleotides (Hirsch and Kaplan, 1990). Prolonged treatment of experimentally infected mice with acyclovir does not ablate latency (Field *et al*, 1979, Blyth *et al*, 1980; Field and De Clercq, 1981; Clements and Subak-Sharpe, 1988).

Viral DNA in the trigeminal ganglia of latently infected mice is in a different physical state to the linear form present in virions and acutely infected cells (Rock and Fraser, 1983, 1985; Puga *et al*, 1984). In particular, latent genomes lack detectable termini, consistent with circular, catenated, or concatameric molecules,

or integration into the host cell genome. Efstathiou and colleagues (1986) confirmed and extended these findings by showing that (i) viral genomes lacking termini are found in latently infected human trigeminal ganglia and (ii) all four HSV DNA isomers were present in latently infected tissues. Latent viral DNA probably exists in an episomal, unintegrated form as shown by buoyant density centrifugation (Mellerick and Fraser, 1987) and is associated with nucleosomes in a structure similar to eukaryotic chromatin (Deshmane and Fraser, 1989).

Latency may not be entirely static in nature. Klein (1985) hypothesised that spontaneous reactivations repeatedly replenish the reservoir of latent infection. However, experimental superinfection of ganglia is inefficient (Centifanto-Fitzerald *et al*, 1982; Meignier *et al*, 1983) suggesting that a 'round trip' phenomenon is unlikely to be of major importance.

1.5.6 The amount of viral DNA present in latently infected tissues

Estimates from quantitative blot hybridizations range from 0.1 to 1 copy of the HSV genome per cell in latently infected sensory ganglia from mice and humans (Puga *et al*, 1978; Cabrera *et al*, 1980; Rock and Fraser, 1983; Efstathiou *et al*, 1986). Because only a small proportion (less than 10%) of all cells in these tissues are neurons (Pannese, 1964; Pannese *et al*, 1972, 1973; Walz *et al*, 1976) and because less than 10% of neurons, as shown by infectious centre assays (Walz *et al*, 1976; Croen *et al*, 1987) and *in situ* hybridization studies, are latently infected, it follows that each neuron harbouring latent HSV contains many copies

of the viral genome. Stevens (1989) estimates that there are 20 copies of the HSV genome per latently infected neuron while Roizman and Sears (1987) suggest the number could be as high as several hundred. To date, there have been no reports of detection of latent DNA by hybridization *in situ*, and consequently direct measurement of latent DNA copy number using quantitative *in situ* techniques has not been possible.

In view of the dependence of viral DNA replication on several viral gene products (Challberg, 1986; McGeoch *et al*, 1988b; Wu *et al*, 1988). The apparent presence of multiple copies of the viral genome in latently infected neurons is difficult to reconcile with evidence, derived from studies on cultured cells, that viral replication is invariably cytolytic (Roizman and Sears, 1990). There are a number of ways in which these conflicting data might be resolved. First, estimates of the fraction of ganglionic cells that harbour viral genomes might be low. Second, more than one viral genome might enter each neuron during the establishment phase. Third, HSV DNA may be amplified either as a result of abortive infection (Simmons and Tscharke, 1992) or as a result of interaction between viral genomes and host cell enzymes, as suggested by Sears and Roizman (1990).

1.5.7 Viral functions necessary for establishment of latency

Studies defining specific viral functions associated with establishment of latent infection *in vivo* are complicated by concurrent productive infection (Stevens, 1989). Consequently viruses containing defined mutations that interrupt

productive infection have been widely used in the search for viral functions pertaining to latency. Several types of mutant virus have been studied:

1. Temperature-sensitive mutants: Temperature-sensitive (ts) mutants of HSV which are replication-defective at the core temperature of mice (38.5°C) retain the ability to persist in the nervous system, suggesting that viral replication is not essential for establishment of latency (Lofgren *et al*, 1977; McLennan and Darby, 1980; Watson *et al*, 1980). This conclusion is weakened by the possibility that ts mutants may spontaneously revert to wild type (leakiness), and it is further complicated by the fact that viral mutagenesis can introduce silent mutations in non-essential genes (Dargan and Subak-Sharpe, 1984). However, despite possible shortcomings of experiments with ts mutants, the conclusions drawn have been confirmed by other approaches.

2. Thymidine kinase mutants. The behaviour of viruses with mutations or deletions in this gene (TK^- viruses) has been carefully scrutinized. Early studies showed that TK-defective viruses multiply at the site of inoculation but cannot be reactivated by explant culture of latently infected ganglia (Tenser and Dunstan, 1979; Tenser *et al*, 1979), leading to speculation that expression of this gene is required for establishment of latency. However subsequent studies (Efstathiou *et*

al, 1989) showed that TK$^-$ HSV can be recovered from latently infected ganglia by superinfection and LAT expression is evident in ganglionic neurons of animals infected in the periphery with TK$^-$ viral mutants (Coen *et al*, 1989; Leist *et al*, 1989). This is consistent with the view that thymidine kinase expression is important in systems used to detect reactivation and not in the establishment of latency.

3. α gene mutants. Viruses with lesions in essential α genes such as α4 and α27 (Leib *et al*, 1989b) are, like replication-defective ts mutants, capable of establishing latency in animals.

4. VP16 mutants. The tegument protein VP16 transactivates α genes (Post *et al*, 1981; Batterson and Roizman, 1983; Campbell *et al*, 1984; Pellett *et al*, 1985; Marsden *et al*, 1987), initiating the cascade of gene expression associated with lytic infection. Inactivation of the transactivating function of VP16 (Ace *et al*, 1989) does not interfere with persistence of the viral genome, suggesting that initiation of α gene expression is not a requirement for establishment of latent infection.

In summary, despite intense scrutiny of the biological behaviour of many replication-defective mutants, no viral function specific for latency has been identified. This has been interpreted as showing that latency and lytic infection

diverge at an early stage and that the minimum requirements for establishment of latent infection do not include expression of any virally-encoded gene. It is not clear whether wild type viruses, in which entry into the lytic pathway is not precluded by a lesion in the viral genome, behave in the same way as mutants.

1.5.8 Viral activity during latency

During latency there is limited transcription of the viral genome (Galloway *et al*, 1979; Tenser *et al*, 1982; Galloway *et al*, 1982; Stroop *et al*, 1984; Stevens *et al*, 1987). Two or three colinear latency associated transcripts (LATs), in the range 1.45-2.0 kb (major LATs), are detected by Northern blot analysis of RNA extracted from latently infected ganglia of humans and experimental animals (Stevens *et al*, 1987; Rock *et al*, 1987b; Spivack and Fraser, 1987, 1988b; Steiner *et al*, 1988; Wechsler *et al*, 1988b; Mitchell *et al*, 1990b). Major LATs, which are confined to neuronal nuclei *in situ*, partially overlap and are complementary to the 3' end of α0 mRNA (Deatly *et al*, 1987; Stevens *et al*, 1987; Krause *et al*, 1988; Wechsler *et al*, 1988 a&b). Studies carried out on human cadavers found that seropositivity coincided with detection of LATs *in situ* in trigeminal ganglia, consistent with LATs being a consequence of HSV infection of the natural host and not an experimental artifact (Stevens *et al*, 1988).

1.5.9 LATs are not unique to HSV-1

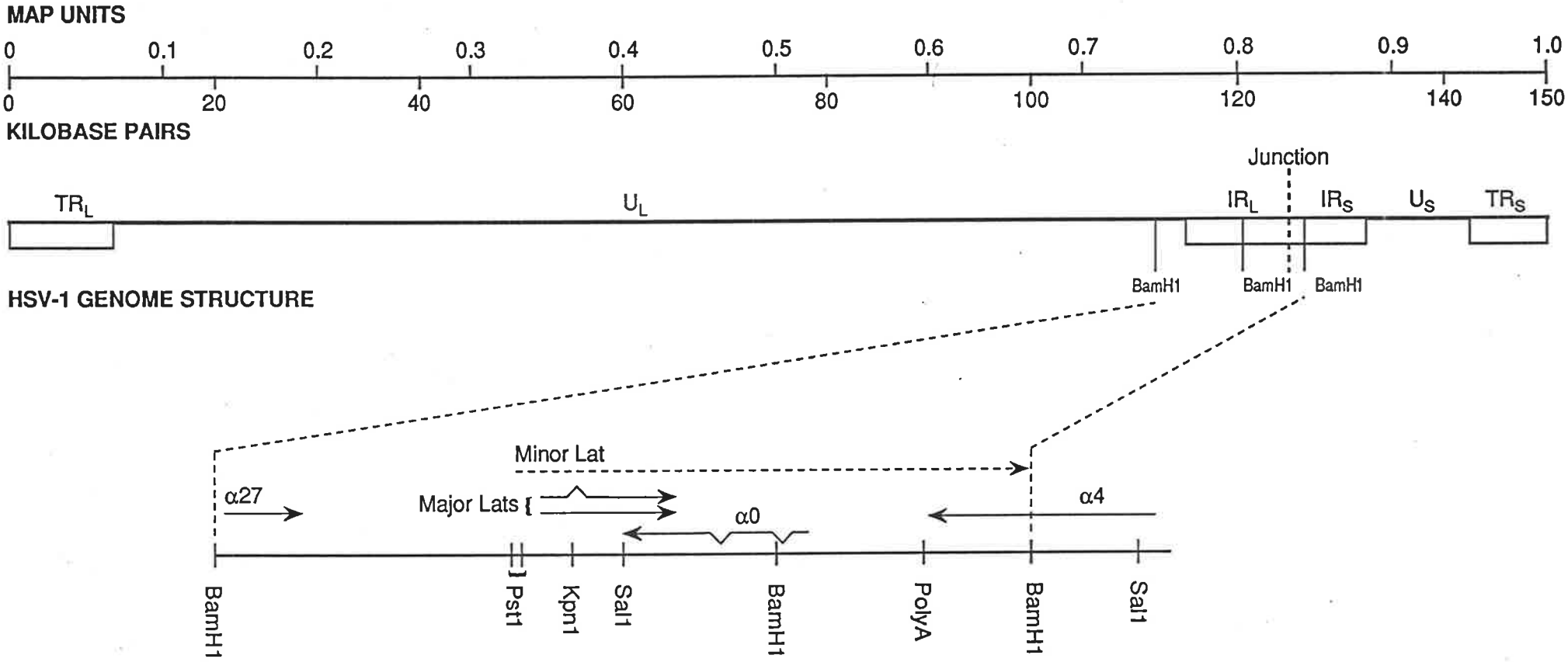
HSV-2 has a pattern of transcription in latency closely resembling that of HSV-1 (Mitchell *et al*, 1990a; Krause *et al*, 1991; Tenser *et al*, 1991). Further, two

neurotropic herpesviruses of animals are known to produce latency related transcripts. Pseudorabies virus, an alphaherpesvirus of swine, establishes latent infection during which polyadenylated RNAs of 2.0 and 0.95 kilobases have been detected (Cheung, 1989). These transcripts map to the same region as the single immediate-early gene of PRV and are of opposite polarity to, and overlap the 3' end of its mRNA. Bovine herpesvirus 1 is an alphaherpesvirus that causes disease in cattle and establishes latent infection in neurons (Homan and Easterday, 1980, 1983). Using a rabbit model, transcripts of approximately 1 kb in length, from a region of BHV-1 that is abundantly transcribed with immediate- early kinetics have been detected during latency (Rock *et al*, 1986, 1987; Kutish *et al*, 1990).

1.5.10 Characterization of latency associated transcription

Whilst characterization of HSV transcripts produced in latency is incomplete, a complex pattern of splicing and processing is emerging (Wagner *et al*, 1988b). In animals latently infected with HSV strain KOS(M), two colinear major LATs, ~2.0 kb and ~1.5 kb, are prominent (Wagner *et al*, 1988a). A third variant, 1.45 kb, has been described in mice infected with strain F (Spivack and Fraser, 1987, 1988b). The 5' ends of these transcripts are located 510 bases to the left of a Kpn I site at 0.783 map units (Wagner *et al*, 1988a) (Fig 1.4). The 3' ends are complementary to the 3' end of α0 mRNA and map to a 310 base-pair SmaI fragment of the viral genome, 660 to 970 base pairs to the right of a Sal I site at 0.790 map units. The nearest RNA polymerase II promoter elements lie in a

Figure 1.4. Map of the HSV-1 genome structure showing the region from which LATs are transcribed. Major LATs (~1.5-2.0 kb) are shown as solid lines. Minor LAT refers to regions reported to be transcriptionally active during latency as detected by *in situ* hybridization. (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; Poly A: first polyadenylation site 3' of major LAT region).


MAP UNITS
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
20
40
60
80
100
120
140
150
KILOBASE PAIRS
Junction
TRL
UL
IRL
IRS
US
TRS
BamH1
BamH1
BamH1
HSV-1 GENOME STRUCTURE
Minor Lat
α27
Major Lats
α0
α4
BamH1
Pst1
Kpn1
Sal1
BamH1
PolyA
BamH1
Sal1

region spanning from 500-1000 nucleotides upstream of the 5' end of major LATs. Contained in this region is a TATA box, a CAAT box and three Sp1-binding sites, or GC boxes (Jones and Tijan, 1985; Kadonaga *et al*, 1987) which makes this region an excellent candidate for a transcriptional promoter (Wechsler *et al*, 1988b). In transient expression assays this region displays strong promoter activity (Dobson *et al*, 1989; Zwaagstra *et al*, 1990) with neuronal selectivity (Batchelor and O'Hare, 1990). Deletion of this TATA box ablates synthesis of LATs (Dobson *et al*, 1989). These data strongly suggest that the polymerase II promoter element ~700 nucleotides upstream of the 5' end of major LATs is required for transcription during latency. There are three potential explanations for this apparently ususual relationship between major LATs and their promoter:

1. Major LATs may be stable introns derived from a larger, less abundant transcript (Dobson *et al*, 1989; Farrell *et al*, 1991). In support of this proposition, a wide region (~10 kb) of the viral DNA is positive for transcripts by *in situ* hybridization, corresponding to the region between the LATs promoter and the vicinity of the first polyadenylation signal encountered downstream. Further, an ~8.3 kb transcript from the same region has been detected in productively infected cells (Dobson *et al*, 1989; Devi-Rao *et al*, 1991) and (in one report) in latently infected ganglia (Zwaagstra *et al*, 1990). Finally, the DNA sequence surrounding the major LATs contains consensus splice acceptor and donor sites (Wagner *et al*, 1988b) which are

operative when LATs are transcribed during neuronal latency (Farrell *et al*, 1991).

2. If LATs are not introns, they may have a short leader sequence. There is no evidence to support the existence of a 5' leader sequence, though it is possible that very short sequences might not be detected by Northern blot analysis. An example of a short 5' leader sequence is the leader of the adenovirus major late transcript (Akusjarvi and Pettersson, 1979; Zain *et al*, 1979).

3. Transcription may initiate ~700 nucleotides from the promoter. There are several genes in which the nearest TATA box and CAAT box are several hundred nucleotides upsteam of the transcription start site. These genes include those for hypoxanthine phosphoribosyl-transferase (Stout and Caskey, 1985) and adenosine deaminase (Valerio *et al*, 1985). Genes lacking TATA boxes near transcription start sites may initiate at more than one site (Benoist and Chambon, 1981; Maue *et al*, 1990). By analogy, the transcription observed during latent HSV-1 infection may be from a single promoter by initiation at more than are site. Transcription from an upsteam site(s) would account for the minor *in situ* hybridization (Deatly *et al*, 1987, 1988; Steiner *et al*, 1988; Mitchell *et al*, 1990a, 1990b) and transcription from a downstream site would account for the LATs detectable by Northern

blotting (Rock *et al*, 1987b; Spivack and Fraser, 1987; Stevens *et al*, 1987). Existence of a neuron specific regulatory element (CCAGG: Maue *et al*, 1990) close to the 5' end of major LATs supports this proposal. An RNA polymerase IIIB promoter element is also found in the vicinity of the 5' end of major LATs (Wagner *et al*, 1988a) but there is no evidence that it directs transcription.

The LATs promoter, in transient expression assays, is efficiently repressed by ICP4 and this effect is dependent on a 55 base pair sequence within the LATs promoter. The LATs promoter contains a cyclic AMP responsive region 12 bases upstream of the TATA box leading to speculation that cyclic AMP may act in viral reactivation (Leib *et al*, 1991).

The complicated splicing pattern of transcripts in the LAT region contrasts to the majority of HSV transcripts which are unspliced (Roizman and Sears, 1990).

Latency associated transcription of HSV-2 is similar to that of HSV-1 although it is not as well characterised. Northern and *in situ* hybridization studies show a single major HSV-2 LAT species of 2-3 kilobases in length and also show that up to 11.5 kilobases of DNA may be transcribed during latency (Mitchell *et al*, 1990b). Genomic location (overlapping the 3' end of α0 mRNA) is similar for HSV-1 and HSV-2 LATs.

1.5.11 Potential functions of major LATs

The role of transcription during establishment, maintenance and reactivation of latency has been examined using HSV mutants containing deletions or disruptions in the promoter and/or in the 5' region of major LATs (Javier *et al*, 1988; Ho and Mocarski, 1989; Izumi *et al*, 1989; Leib *et al*, 1989a, 1989b; Sederati *et al*, 1989; Steiner *et al*, 1989; Hill *et al*, 1990; Block *et al*, 1990). At least one such mutant virus has been shown to have no alteration in virulence or in ability to replicate in mouse cells *in vivo* and *in vitro* compared with parental virus (Izumi *et al*, 1989). LAT mutants so far constructed establish, stably maintain and reactivate from latent infections showing that expression of LATs is not an absolute requirement for these processes. However LAT^- viruses reactivate with slower kinetics or less effectively than parental viruses, suggesting that LATs may increase the efficiency of reactivation (Leib *et al*, 1989a; Steiner *et al*, 1989; Hill *et al*, 1990).

In vivo, spontaneous and experimentally induced reactivation may differ in their dependence on LATs. In the rabbit eye model reactivation occasionally occurs spontaneously or can be induced by iontophoresis of adrenalin into the cornea (Hill *et al*, 1987, 1990). The rate of spontaneous reactivation is not altered by deletion of LATs. In contrast, induced reactivation in this model is LATs dependant.

It is not known whether LAT^+ neurons represent all sites of latency or whether

LAT^+ neurons are the sole reservoir from which virus reactivates. Tenser and Hyman (1987) provided circumstantial evidence of a relationship between LAT^+ sites and reactivation based on equivalent numbers of LAT^+ neurons in latently infected ganglia of mice and antigen-expressing neurons during reactivation *in vitro*.

There are several potential ways in which LATs might influence the biology of herpes simplex.

1. In transient transfection assays, LATs are able to inhibit expression of α0 mRNA (Farrell *et al*, 1991) and therefore it has been suggested that LATs might downregulate the activity of the α0 gene *in vivo*, by complementary binding to its mRNA (Stevens *et al*, 1987). However, α0 mRNA cannot be detected during latency even using the polymerase chain reaction technique (Lynas *et al*, 1989). Further, LAT^- mutants establish and maintain latency in a manner indistinguishable from wild type virus.

2. Latent HSV genomes are associated with nucleosomes (Deshmane and Fraser, 1989) but this association would not extend to an area of the genome that continues to be transcribed. Thus LATs may function by keeping a region of the viral genome accessible to transcription factors, facilitating expression of lytic genes during reactivation.

3. Major LATs might encode a protein of hitherto unknown functions. Analysis of the nucleotide sequence of HSV-1 major LATs has shown two potential open reading frames (ORFs) but the nuclear location and lack of polyadenylation of major LATs suggest they do not function as mRNAs. Further, whilst conservation of ORFs between strains of HSV-1 is strong (Wagner *et al*, 1988a; Wechsler *et al*, 1989), there is little conservation of LAT ORFs between HSV-1 and HSV-2 (Mitchell *et al*, 1990b) or between HSV-1 and PRV (Cheung, 1989, 1990). Whilst there is a consistent scheme of transcription during latency for HSV-1, HSV-2, pseudorabies virus and bovine herpesvirus type 1, it is unlikely that this similarity extends to expression of a common protein. In addition, there are at least 2 reports of antisera raised against polypeptides potentially encoded by the major LATs region being unsuccessful in the search for a protein product (Wagner *et al*, 1988a; Wechsler *et al*, 1989) in latently infected ganglia. Nevertheless an antiserum directed against a bacterially expressed fusion protein containing part of a putative HSV-1 LATs encoded polypeptide recognizes a latency associated antigen (LAA) in neuronally derived cells *in vitro* (Doerig *et al*, 1991a). Western blot analysis suggests that the LAA is an 80 KDa protein, whereas the larger of the two major LATs ORFs potentially encodes a protein of only 35 KDa molecular weight.

1.5.12 *In vitro* systems of latency

Whilst animal models have provided essential information about latency, molecular characterization has been limited by the low number of cells in sensory ganglia that harbour viral genomes and the diverse cell types present in neural tissues. Considerable effort has therefore been directed towards development of systems in which latency is reproduced in cultured cells. In initial experiments, viral DNA was shown to persist in cells infected at supraoptimal temperatures and/or in the presence of inhibitor of viral replication (O'Neill, 1977; Wigdahl *et al*, 1981, 1982, 1983; Shiraki and Rapp, 1986). Preston and Russell (1991) found that viral DNA persists in a non-linear form, resembling that found *in vivo*, in cells infected at 42°C at low multiplicity (<0.03 plaque forming units/cell). In at least one system LATs have been detected in neuronally-derived cells harbouring HSV genomes *in vitro* (Doerig *et al*, 1991b). It is therefore likely that *in vitro* systems will contribute substantially to the further characterization of established latency at the molecular level. However, at this time experimental models of infection *in vivo* are optimal for studying the dynamic processes associated with the establishment phase.

1.6 Aim of project

Viruses that have been denied the possibility of initiating a lytic infection, for example, by deletion of an essential gene such as that encoding ICP4, retain the ability to persist in the host (see 1.5.7), from which it has been concluded that the pathways of latent and productive infection are divergent from a very early stage.

A corollary of this conclusion, taking into account the belief that replication of the HSV genome is dependent on several virally encoded proteins, is that viral DNA cannot be amplified during the establishment of latency. This corollary is not supported by indirect assessment of latent DNA copy number following infection with replication-competent viruses (sec 1.5.6).

Consequently, deletion mutants may not accurately represent the behaviour of replication-competent viruses. Therefore, in this project, novel use was made of the segmental innervation of mouse flanks to study molecular events associated with establishment of latency following inoculation with a virulent strain of HSV-1. The aim was to determine whether virus that is not compromised in its ability to enter the lytic pathway by a defect in its genome establishes a latent infection without accompanying viral gene expression.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Virus stocks

Experiments were performed with a well characterized recent oral isolate of HSV-1, strain SC16 (Hill *et al*, 1975). The pathogenicity of this strain of HSV-1 has been carefully studied by various routes of inoculation with several mouse strains (Harbour *et al*, 1981; Tullo *et al*, 1982; Blyth *et al*, 1984; Simmons and Nash, 1984, 1985, 1987; Nicholls and Blyth, 1989; Simmons and La Vista, 1989; Simmons, 1989; Scalzo *et al*, 1990). Strain SC16 is moderately neurovirulent after cutaneous inoculation and produces a transient phase of productive infection in spinal ganglia followed by a stable latent infection that is indistinguishable at the molecular level from that seen in humans (Efstathiou *et al*, 1986). Fresh working stocks, made at regular intervals from sub-master stocks, were grown and titrated in Vero cells and stored at -70°C until required. All master and sub-master stocks were stored in liquid nitrogen.

2.1.2 Mice

Female BALB/c and C57BL10 mice were obtained from the Animal Resource Centre, Gilles Plains, South Australia and the Animal Resource Centre, Perth, Western Australia, respectively. All mice were used at greater than 6 weeks of age. Mice were bred under specific pathogen-free conditions and the genetic authenticity of the strains was checked and confirmed by the suppliers at 6

monthly intervals.

2.1.3 Plasmids used for production of riboprobes

pBS0 and pBS4 were gifts from Dr S Efstathiou, Cambridge University, UK. pBS0 contains a 2557 bp BamH1/SalI fragment from HSV-1 strain KOS, spanning map units 0.790 to 0.807 (Fig 1.4) inserted into Bluescript M13$^-$ (Stratagene Cloning Systems). Transcripts from the T7 promoter of pBS0 are complementary to major LATs and T3 transcripts are complementary to α0 mRNA. pBS4 contains a 1786 bp BamH1/SalI fragment from HSV-1 strain KOS inserted into Bluescribe M13$^-$. Transcripts from the T7 promoter are complementary to α4 mRNA.

pBAZ1, a gift from B Slobedman, Institute of Medical and Veterinary Science, Adelaide, contains an 875 bp Pst1 fragment of the thymidine kinase gene of HSV strain F, inserted into Bluescribe M13$^-$. Transcripts from the T7 promoter are complementary to viral thymidine kinase mRNA.

Plasmid TM46, a gift from Dr T Macnaughton, Institute of Medical and Veterinary Science, Adelaide, contains the genome of hepatitis B virus strain CB, inserted into expression vector pGEM 3B (Promega).

2.1.4 Buffers and solutions

HDMEM: HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) -

buffered Dulbecco modified Eagle's medium. (Gibco BRL).

PLP: Periodate-lysine-paraformaldehyde:

0.01M Na periodate

0.075M lysine

2% paraformaldehyde

0.037M phosphate buffer, pH 7.4

(McLean and Nakane, 1974).

PBS: Phosphate buffered saline, pH 7.4 (Sambrook *et al*, 1989).

Decalcification solution, pH7.4 (Brain, 1966):

30 mM NaH_2 PO_4

70 mM Na_2H PO_4

200 mM Na_2 EDTA

5x Transcription Buffer:

200 mM Tris HCl pH 7.5

30 mM $MgCl_2$

10 mM Spermidine

50 mM NaCl

Toluene-based liquid scintillant:

AR-grade toluene, 0.4% POPOP (1,4 - bis [5-phenyl-2-oxazolyl] benzene; 2,2'-p-phenylene-bis-[5-phenyloxazole], Sigma), 0.04% PPO (2,5,-diphenyl-oxazole, BDH).

5x Hybridization buffer:

10 x SSC

500 mM Tris-HCl pH 7.6

50 mM NaH_2 PO_4

50 mM Na_2H PO_4

0.1% Ficoll

0.1% Polyvinyl pyrrolidone

Hybridization mix:

50% deionised formamide (Davis *et al*, 1986)

1xSSC

1x hybridization buffer

0.5 mg/ml sheared denatured salmon sperm DNA

0.5 mg/ml tRNA

20 mM DTT

1 u/µl RNAsin (ribonuclease inhibitor, Promega)

40 pg/µl RNA probe

1x SSC: 0.15 M NaCl

0.015M Trisodium citrate

1x TE: 10 mM Tris-HCl pH 7.5

1 mM EDTA

2.2 Methods

2.2.1 Quantification of infectious virus

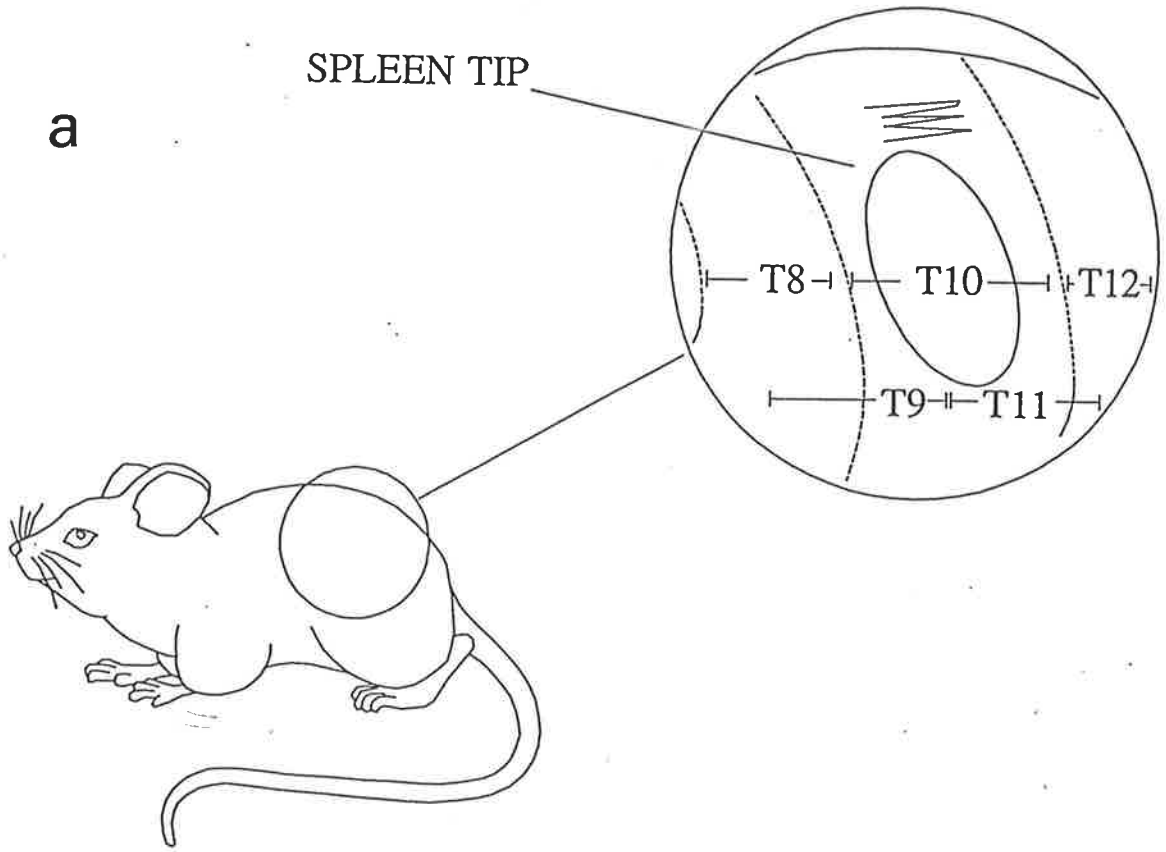
Plaque assays were performed in duplicate by the suspension method of Russell (1962). Vero cells, $3x10^6$ in 100 µl of HDMEM containing 1% fetal calf serum (FCS), were added to serial (tenfold) dilutions of tissue homogenates or virus suspension in 0.9 ml of the same medium. After 1 hour of incubation at room temperature with shaking, 4 ml of HDMEM supplemented with 10% FCS and 1% carboxymethylcellulose was added to each sample. Mixtures were plated in 6 cm plastic dishes and incubated at 37°C for 2.5 days in a humidified 5% CO_2 atmosphere. Cell monolayers were fixed with 10% formalin for 20 mins, washed briefly with tap water, stained with 0.1% toluidine blue for 30 mins and plaques were counted using a low-power dissecting microscope.

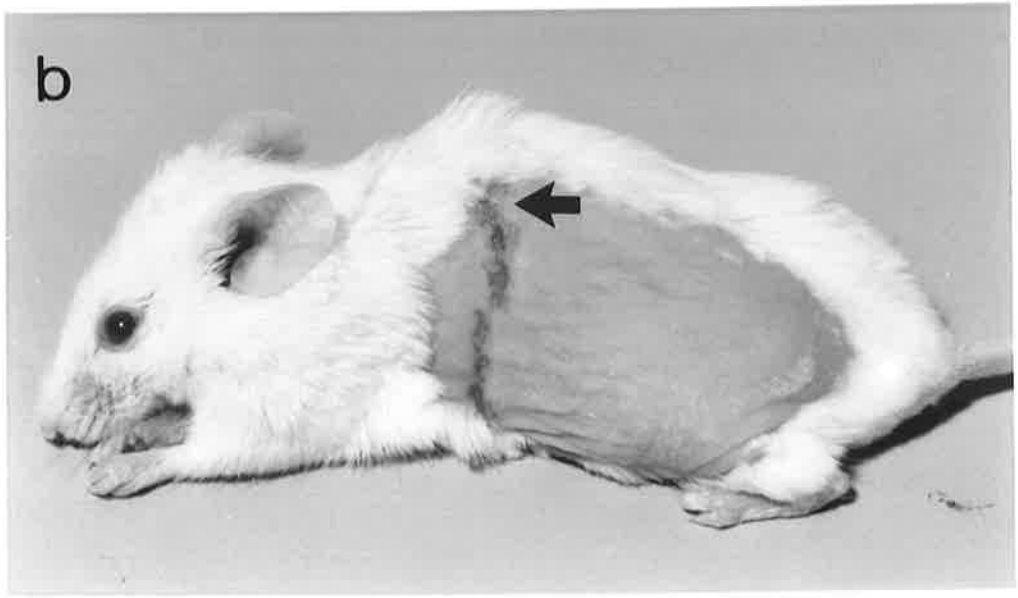
2.2.2 Infection of mice

Left flanks were depilated with Nair (Carter Wallace) prior to infection. Using a 27g needle, skin was scarified 20 times over the spleen tip (Fig 2.1a) through a 10 µl drop of virus suspension containing $3x10^4$ PFU of HSV-1 strain SC16

Figure 2.1. (a) Schematic diagram illustrating location of inoculation site. The inset shows the area of scarification in relation to the spleen, which is visible through depilated skin in the tenth thoracic dermatome (T10). Successive dermatomes overlap their neighbours by approximately 50%. Thus T9 overlaps T8 rostrally and T10 caudally, and T9 and T11 meet around the middle of T10. Consequently any scratch on the flank skin provides direct access to at least three sensory dorsal root ganglia.

(b) Zosteriform lesion on the left flank of a mouse five days after infection by scarification of the skin at one point (arrow).


a
SPLEEN TIP
T8
T10
T12
T9
T11


b

(Simmons and Nash, 1984; A. Simmons, PhD Thesis, University of Cambridge, 1985; Simmons and Nash, 1985; Simmons and La Vista, 1989; Scalzo *et al*, 1990). Infected mice developed a prominent band-like zosteriform lesion on the left flank 5-6 days after inoculation with virus (Fig 2.1b).

2.2.3 Removal of infected tissue from mice

Mice were humanely killed by CO_2 asphyxiation or by intraperitoneal injection of 100 µl of sodium pentobarbitone (60 mg/ml).

The viscera were removed through an anterior midline incision, exposing the anterior surface of the spinal column. The thirteenth thoracic vertebra was identified by its articulation with the thirteenth (lowest) rib. Fine forceps were used to tease apart intervertebral discs and remove the sixth thoracic (T6) to first lumbar (L1) dorsal root ganglia. Sections of spine were removed after exposing the anterior surface of the spinal column and dissecting away the musculature and skin attached to the vertebrae.

2.2.4 *In vitro* reactivation of latent infection

Latent HSV infection was reactivated by culturing neural tissue *in vitro* using well-established techniques (Stevens and Cook, 1971). Briefly, dorsal root ganglia were incubated in 1 ml of HEPES buffered DMEM/1% fetal calf serum in a humidified 5% CO_2 atmosphere for 8 days, homogenised in Wheaton (Millville, New Jersey) tissue homogenisers and tested for the presence of

infectious virus (section 2.2.1).

2.2.5 Fixation of tissues

Dorsal root ganglia were fixed for 60-120 mins in freshly prepared PLP. PLP was chosen for its suitability for preservation of nucleic acids and antigens (McAllister and Rock, 1985; Moench *et al*, 1985; Puchtler and McLoan, 1985; Raymond and Leong, 1988). Fixed dorsal root ganglia were pooled by rolling into a ball.

Spinal cords were fixed *in situ*. After opening the thoracic cavity, a catheter (a blunted 17g hypodermic needle) was inserted through the left ventricle into the aorta and clamped in place. To dilate blood vessels 150 mM $NaN0_2$ in PBS was introduced under pressure of 80-120 mm Hg, exerted using compressed air over the reservoir of fixative. Subsequently, animals were perfused with PLP for 30 mins. A trace of toluidine blue added to the fixative enabled the success of the procedure to be gauged by the colour of the perfused tissue. After tissue removal, sections of spine were clamped between 2 foam rubber sponges pre-wetted with fixative and immersed in PLP for a further 24 hours. Tissues were stored in 50% ethanol until required.

2.2.6 Tissue processing

The production of coronal sections of spine necessitated decalcification of bone by prolonged immersion in decalcification solution. Progress of decalcification

was assessed by taking X-ray photographs using Kodak SO-177 Ortho M Film in a Hewlett Packard Faxitron 43805N machine (60 kV, 2.5 mA; exposure time: 18 seconds).

Tissue specimens were impregnated with paraffin in a Shandon processing machine in the Division of Tissue Pathology, Institute of Medical and Veterinary Science, Adelaide. Gradual dehydration over several hours using graded ethanol solutions was necessary to avoid shrinkage of neurons.

2.2.7 Preparation of slides and coverslips and cutting of sections

Slides were placed in sulphuric acid (10%)/potassium dichromate (0.35M) mixture for 16 hours, washed in 4 changes of DDW, air dried, dipped in 2% aqueous aminopropyltriethoxysilane (APES) for 5-10 seconds, dried and stored for up to one month before use. Immediately before sections were collected slides were activated by immersion in 10% glutaraldehyde for 30 minutes (Maples, 1985). Ganglionic sections (5μ) were cut from paraffin blocks each containing 30-50 pooled ganglia, to yield in total ~200 slides/block. The number of individual ganglionic sections on each slide ranged from 10 through 21.

Coverslips used in ISH were acid washed as above, siliconized by dipping in 1% Prosil (PCR Inc, Gainesville, Florida), rinsed twice in DDW, dried at 80°C for 2 hours, then heat-sterilized.

2.2.8 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 antibodies were (i) rabbit antiserum to HSV-infected cells (Dakopatts, Glostrup, Denmark), (ii) monospecific rabbit antiserum to ICP8 (a gift from Dr K Powell, Wellcome Research Laboratories, Beckenham, United Kingdom), and (iii) monoclonal antibody 58-S (American Type Culture Collection HB8183) to ICP4. Binding of primary antisera was detected using swine anti-rabbit or goat anti-mouse immunoglobulin as appropriate, followed by rabbit or mouse peroxidase-anti-peroxidase conjugate, respectively (all from Dakopatts). All reactions were allowed to proceed for 30 min at 37°C with a 10-min 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), and sections were slightly counterstained with hematoxylin.

2.2.9 *In situ* hybridization

2.2.9.1 Choice of probe

Recombinant DNA probes were selected because they are less likely to contain contaminating sequences than even highly purified naturally derived nucleic acids (Angerer *et al*, 1987). Riboprobes (RNA probes) were used throughout because, being single-stranded, there is less tendency to self-anneal which effectively increases probe concentration, thereby maximising sensitivity (Angerer *et al*, 1987). Further, in some cases opposite sense transcripts can be used as controls

for non-specific binding (Gowans *et al*, 1989).

2.2.9.2 *Choice of indicator molecule*

Four radioactive indicator molecules are in common use in ISH: ^{35}S, ^{3}H, ^{32}P and ^{125}I. ^{35}S, ^{3}H and ^{32}P emit β particles of energy of 167, 18.5 and 1710 keV respectively. ^{125}I has a complex decay pattern producing extra-nuclear electrons with energies of 2.77 keV (27.7%), 3.6 keV (48.8%), 22.5 keV (14.2%), 31.0 keV 6.7% and 34.2 keV (1.2%), and the appearance of grains in autoradiographic nuclear emulsion with this isotope depends on low energy secondary β emissions resulting from ^{125}I decay. Consequently the path length in nuclear emulsion of emissions from ^{3}H, ^{125}I, ^{35}S and ^{32}P are approximately 1μ, 2μ, 10-20μ, and >20μ, respectively (Rogers, 1979). ^{125}I therefore provides much higher resolution in ISH than does ^{32}P or ^{35}S whilst retaining sufficiently high energy to permit exposure times similar to those used with ^{32}P (Gowans *et al*, 1989). ^{3}H was considered to require unacceptably long exposure times. Based on the preceding considerations ^{125}I-CTP (2000 Ci/mM, 1μCi/ul in 70% ethanol) was selected. Despite the 60 day half-life of ^{125}I, it was found that signal-to-noise ratio was compromised if label was used more than 3 weeks after manufacture.

2.2.9.3 *Preparation of riboprobes*

Prior to transcription plasmids were linearised by digestion with a restriction enzyme that cleaved 3' to the cloned fragment insert and samples were electrophoresed on 1% agarose gels to confirm digestion. Transcription reaction

mixtures contained:

4µl 5x transcription buffer

1µl 100 mM DTT

1µl 25 units/µl RNAsin (ribonuclease inhibitor, Promega)

4µl each 2.5 mM ATP, GTP and UTP

2.4µl 100 mM CTP

5µl 10 µCi/µl [α-^{125}I] rCTP (New England Nuclear, Boston)

(2000 Ci/mmol; ^{125}I CTP was dissolved in 1:1 ethanol/H_20 which was dried off under vacuum immediately before use)

1-2 µl (1µg) of linearised template plasmid DNA

1µl (10-15 units) T3 or T7 RNA polymerase (Promega)

H_20 to a final volume of 20 µl.

After incubation at 37°C for 1 hour, 1 unit of RQ1 RNAse-free DNAse (Promega) was added to digest the DNA template and the reaction incubated for a further 15 minutes at 37°C. RNA transcripts were precipitated by the method recommended by the manufacturers of the RNA polymerase (Promega): total volume was brought to 200 µl with RNAse-free H_2O, 10 µl of sodium acetate (3M) and 250 µl of 100% ethanol at -20°C were added. The mixture was stored overnight at -20°C, centrifuged at 13,000 x g for 20 minutes and the pellet washed twice in 100 µl of 70% ethanol to remove unincorporated labelled nucleotides, freeze dried and redissolved in 20 µl of TE.

2.2.9.4 *Measurement of radioactivity and size of RNA transcripts*

Efficiency of incorporation of radiolabel into RNA transcripts was measured by differential precipitation of RNA by trichloroacetic acid (TCA; Sambrook *et al*, 1989). 1 µl samples of reaction mixture were dried onto each of two 1 cm discs of filter paper (Whatman S42). One filter, used to measure total radioactivity in the reaction (i.e. acid-soluble and acid-precipitable radioactivity), was not washed. The other filter was washed in 10% TCA to measure acid-precipitable radioactivity. During washing, RNA molecules more than 50 nucleotides long precipitate onto the filter paper (Sambrook *et al*, 1989) and unincorporated nucleotide precursors elute from the filter. After washing, radioactivity on the filter, dehydrated by successive washes in 100% ethanol, 50% ethanol/50% ether and 100%, was counted in toluene-based liquid scintillant on a Beckman LS6800 counter. Proportion of radionucleotide incorporated was calculated by dividing the counts on the acid-washed filter by the counts on the unwashed filter. Typically, greater than 90% of available radiolabel was incorporated into RNA transcripts.

RNA transcripts were sized by electrophoresis on 1% agarose, 6.6% formaldehyde, Tris-acetate denaturing gels. A sample of the transcript containing approximately 10^5 cpm of labelled RNA was denatured immediately before electrophoresis in 20 µl of 50% formamide/50% electrophoresis buffer for 15 min at 60°C. The mixture was cooled, mixed with 5 µl of formaldehyde gel-loading buffer (Sambrook *et al*, 1989) and electrophoresed. Molecular weight standards

were prepared by digesting pBS4 with restriction enzymes BamH1 and HindIII. The resulting DNA fragments of approximately 1.8 and 3.2 kilobases were end-labelled with [^{32}P]-dCTP and [^{32}P]-dATP by the method of Sambrook *et al* (1989). DNA molecular weight standards provide accurate estimations of the size of single-stranded RNA molecules because they have similar mobility to RNA molecules in denaturing gels (Maniatis *et al*, 1982; Rickwood and Hames, 1982). After electrophoresis, gels were wrapped in plastic and exposed overnight to X-ray film (Kodak, X-Omat RP) which was developed in an Ilfospeed 2240 (Ilford Australia) developing machine. For probes made from pBAZ1, the insert of which is 875 bp in size, the majority of the label was incorporated in full length transcripts. Probes transcribed from the larger plasmids pBS0 and pBS4 were predominantly less than full length and appeared on gel electrophoresis as smears ranging from approximately 1.0 kb to full size transcripts, (average ~1.5 kb).

2.2.9.5 Preparation of tissue sections for ISH

The method used was an adaptation of that of Gowans *et al* (1989). Paraffin sections (5 µm) were dewaxed in xylene, rehydrated gradually through graded ethanol/water mixtures over a period of 1 hour, fixed in 0.1% glutaraldehyde in PBS for 30 min at 4°C, and washed in PBS (2x5 min). Digestion of tissue sections with proteinase K improves probe access but over-digestion results in loss of target sequences and poor morphology (Angerer *et al*, 1987; Gowans *et al*, 1989). The effect of varying proteinase K concentration over a range spanning 10-200 µg/ml was tested. The optimum concentration of proteinase K was 100

µg/ml, at which strong autoradiographic signal was combined with good preservation of tissue morphology. Therefore in all experiments tissue was digested with proteinase K (Merck), 100 µg/ml, in 20 mM Tris-HCl pH 7.4, 2 mM CaCl for 15 minutes at 37°C, washed in PBS, refixed in glutaraldehyde for 15 min, and washed twice more in PBS. For acetylation, sections were treated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes at room temperature (Hayashi *et al*, 1978). Sections were washed twice in PBS, dehydrated gradually and dried prior to application of hybridization mix.

2.2.9.6 Nuclease digestions

After proteinase K digestion, where indicated, tissues were treated for 30 min at 37°C with 100 µg/ml DNAse 1 (Promega) in 40 mM Tris-HCl pH 7.6, 10 mM NaCl, 6 mM $MgCl_2$ or 200 µg/ml RNAse A (Pharmacia) in 2xSSC.

2.2.9.7 Probe specific activity and probe concentration

Recommended specific activity using ^{125}I labelled RNA probes is $5x10^8$ dpm/µg (Gowans *et al*, 1989), calculated as shown in section 2.2.9.13. Lower specific activities, in the range $1.5x10^8$ to $3.7x10^7$ dpm/µg, were associated with a reduction in autoradiographic grains over neurons (data not shown). Background signal in ISH increases linearly as a function of probe concentration (Cox *et al*, 1984) and variations from the recommendation of 40 pg/µl (Gowans *et al*, 1989) spanning a range up to 120 pg/µl were associated with an increase in background autoradiographic grains over uninfected neurons (data not shown). Therefore, all

experiments were done using 40 pg/µl of probes with a specific activity of $5x10^8$ dpm/µg.

2.2.9.8 Hybridization

Hybridization mix (2.5 µl) was applied to sections and covered with a 13 mm siliconised coverslip, taking care not to trap air bubbles. For larger sections the volume of probe mix was increased in proportion to coverslip area. Coverslips were sealed with rubber cement (Vulkarn, Maruni Industries, Osaka) and slides were incubated at Tm -25°C (Cox *et al*, 1984; Gowans *et al*, 1989) for 16 hours (75°C, 79°C or 69°C for probes transcribed from pBSO, pBS4 or pBAZ1 respectively). Salt concentration was chosen to give hybridization temperatures compatible with good tissue preservation.

2.2.9.9 Washing procedure

To remove unbound probe, slides were rinsed in 2xSSC at room temperature, washed once in 2xSSC for 1 hour, and twice in 0.1xSSC for 1 hour at room temperature. High stringency washing was for 20 min in 30% deionised formamide in 0.1xSSC at 75°C, 79°C or 69°C for probes transcribed from pBS0, pBS4 or pBAZ1 respectively. Formamide concentration was chosen to give washing temperatures compatible with good tissue preservation. Potassium iodide (100 mM) was included in all washes to reduce background (McAllister *et al*, 1983). The final wash was done in 0.1xSSC for 30 min at room temperature. Slides were dehydrated through graded ethanol solutions and air dried prior to

dipping in nuclear emulsion.

2.2.9.10 Autoradiography and staining

Slides were dipped in Amersham LM-1 emulsion, set immediately on an ice-cold flat metal plate for 30 mins, dried overnight at room temperature and stored in a light-tight box at 4°C for 2-5 days. Exposed slides were developed in Kodak D-19 developer for 4 minutes at 23°C, with gentle agitation for 5 seconds at 30 second intervals, rinsed in 1% acetic acid stop bath for 30 seconds and placed in Ilford Hypam rapid fixer (diluted 1:4) for 8 minutes. Optimal development time was determined empirically as recommended by Rogers (1979). Slides were washed in tap water for 30 minutes, stained with rapid haematoxylin (Gowans *et al*, 1989) for 30-45 secs and 'blued' in 0.1xSSC for at least 30 minutes. Sections were dehydrated in graded ethanol solutions, washed in xylene (2x10 minutes) and coverslipped using DePex (BDH) mountant.

2.2.9.11 Definition of a LAT$^+$ neuron

Probe hybridization specific for LATs was judged by counting autoradiographic grains using an oil immersion lens (x1000) (Fig 2.2 a-c). Autoradiographic grain counts >3 SD above the mean level for uninfected cells were considered to indicate presence of LATs (>99.75% confidence limits). In uninfected tissue there were approximately 10 grains/neuronal profile/day of exposure (background) (Table 2.1). On this basis, the theoretical lower limit of sensitivity at 3 days exposure is represented by 51.6 grains.

Figure 2.2. Typical appearance of autoradiographic grains (x1000) over sections of latently infected BALB/c spinal ganglia hybridized to ^{125}I labelled LAT-specific transcripts. The neuronal profile in the centre of each field has (a) 85 grains (b) 146 grains (c) 418 grains.

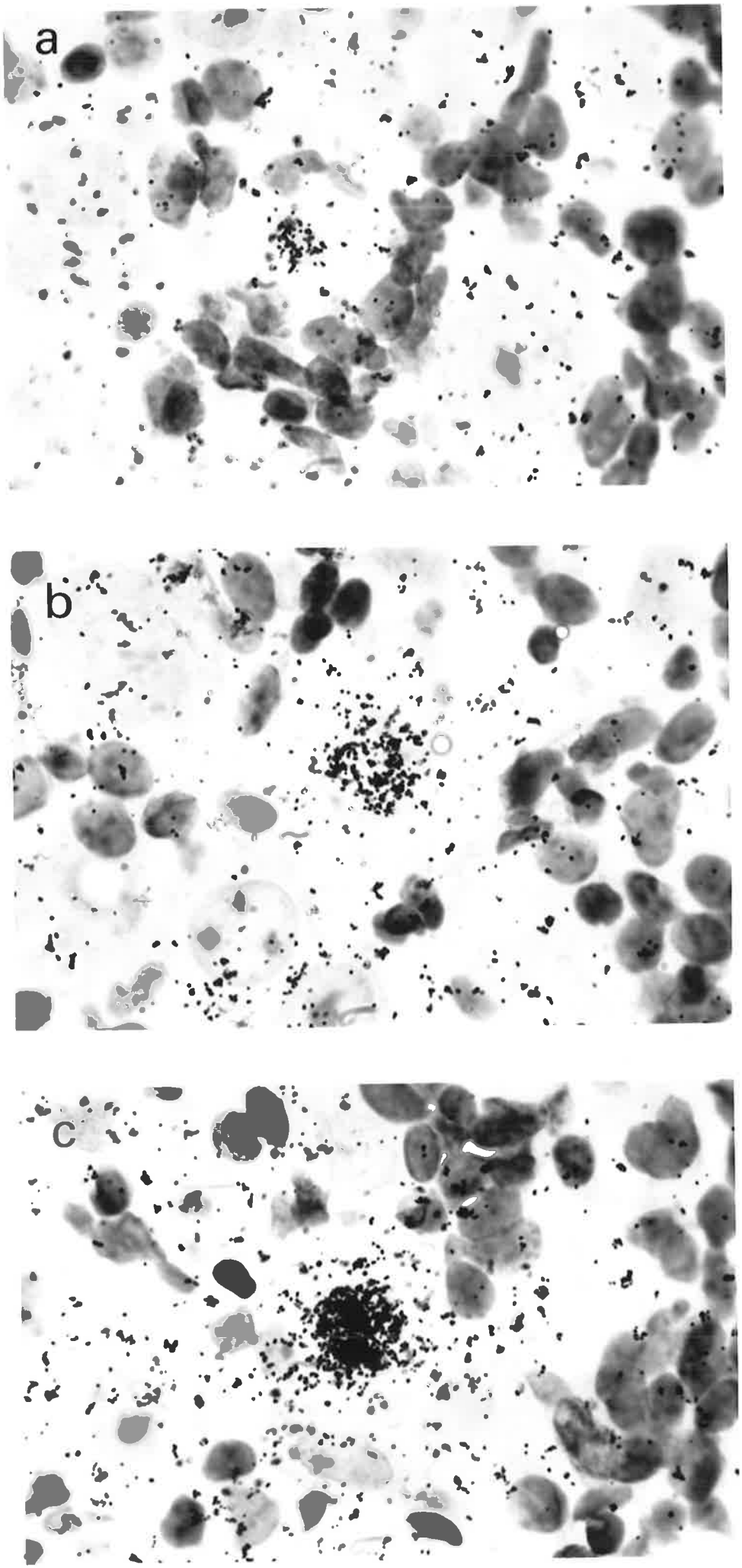

a
b
c

TABLE 2.1

Autoradiographic background: grain counts over sections of uninfected neuronal tissue.

Days of exposure	No. of neuronal profiles counted	$\bar{x}$[1]	SD	$\bar{x}$ + 3(SD)
1	37	9.1 (9.1)	3.8	20.5
3	23	27 (9.0)	8.2	51.6
5	17	53.6 (10.7)	8.3	78.5

$\bar{x}$ = arithmetic mean

SD = standard deviation

[1] Number in brackets is mean number of grains per neuronal profile per day of exposure.

2.2.9.12 Calculation of Tm_{50}

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

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

(Meinkoth and Wahl, 1984; Bodkin and Knudson, 1985).

2.2.9.13 Calculation of specific activity of riboprobe

This is calculated as follows:

G+C content of template	=	75%
specific activity ^{125}I CTP	=	2000 nCi/pmole
1 Ci	=	2220 dpm
ratio of labelled C: unlabelled C	=	1:9 (i.e. 1 in 10)

$\therefore$ there are 110 pmoles ^{125}I C per µg RNA

$\therefore$ calculated specific activity of probe:

$= 110 \times 2000 \times 2220$ dpm/µg

$= 4.88 \times 10^8$ dpm/µg

2.2.10 Dual labelling

Simultaneous detection of nucleic acids and proteins on the same tissue section (dual labelling) (Blum *et al*, 1984; Brahic *et al*, 1984; Gendelman *et al*, 1985; Gowans *et al*, 1989; Hofler *et al*, 1986, 1987; Shivers *et al*, 1986) enables the

presence of viral mRNA to be correlated with the presence of virally encoded proteins. This technique has not been previously applied to the study of HSV.

Dual labelling was carried out by the method of Gowans *et al* (1989) in which immunohistochemistry precedes ISH. As a precaution against degradation of RNA in sections, all immunohistochemical reagents were filter sterilized using 0.2 µm filters (Sartorius) and contained 1 U/µl RNAse inhibitor (Promega) and 1 mM DTT. After immunohistochemistry, sections were washed in 0.1% Triton X-100 in PBS for 10 mins at room temperature to minimize non-specific binding of probe and to increase hybridization efficiency on immunohistochemically stained cells (Gowans *et al*, 1989). Sections were then subjected to ISH as above.

3. VALIDATION OF *IN SITU* HYBRIDIZATION FOR LATs AND DUAL LABELLING

3.1 *In situ* hybridization

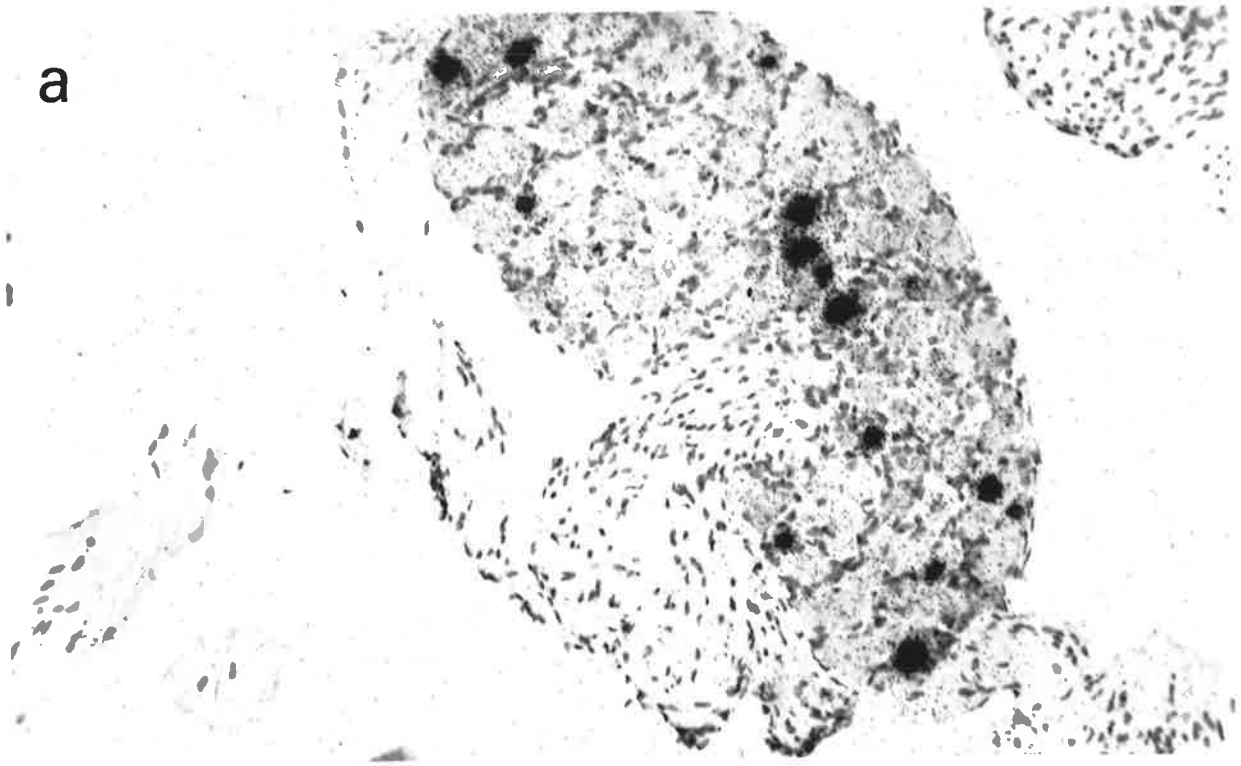
T7 transcripts generated from pBS0 hybridized to latently infected ganglia of BALB/c and C57BL10 mice (Fig 3.1 a&b). Specificity of hybridization was assessed as follows:

(a) Specificity of hybridization for HSV infected tissues was confirmed by showing that T7 transcripts of pBS0 hybridized exclusively to material from latently infected rather than uninfected animals (Fig 3.2 a&b). Sections from uninfected animals were included as negative controls in all *in situ* hybridization experiments.

(b) Specificity of hybridization for HSV nucleic acid sequences was confirmed by showing that an RNA probe (T7 transcripts from plasmid TM46) to irrelevant sequences (hepatitis B virus) did not hybridize to latently infected ganglia (8.1 autoradiographic grains/neuronal profile/day of exposure).

(c) Specificity of the LAT probe for RNA rather than DNA was established in two ways:

Figure 3.1. Detection of LATs by *in situ* hybridization to sections of thoracic ganglia taken from (a) BALB/c mice (magnification, x100) 150d after infection and (b) C57BL10 mice (magnification x250) 120d after infection. The abundance of LATs is illustrated by the dense clusters of grains typically found over neuronal nuclei after 2d photographic exposure.


a

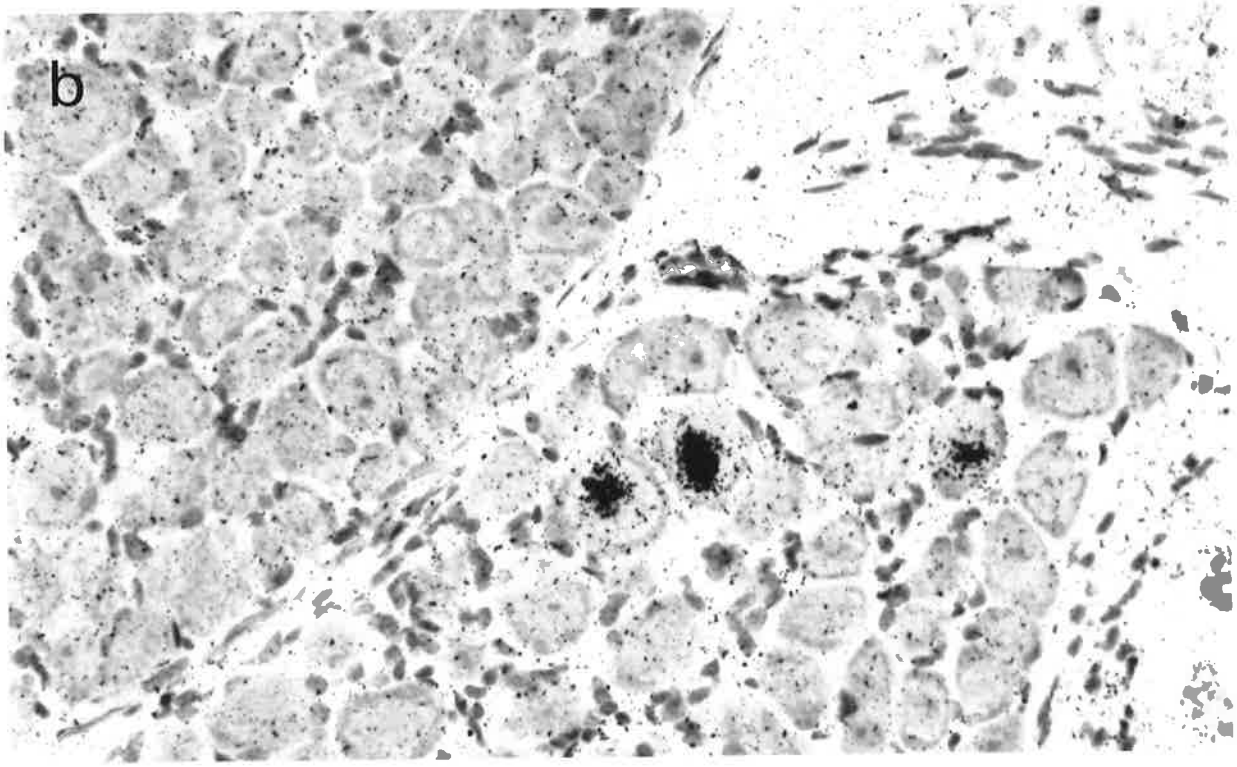
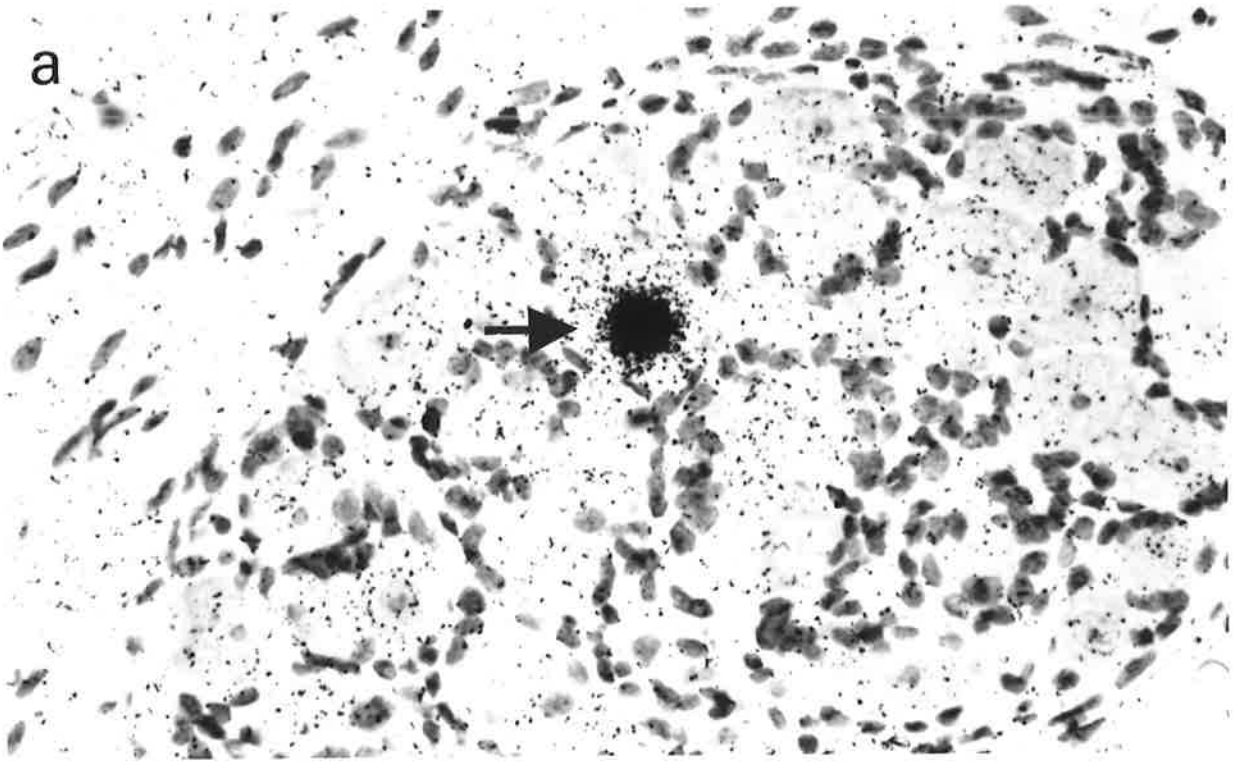
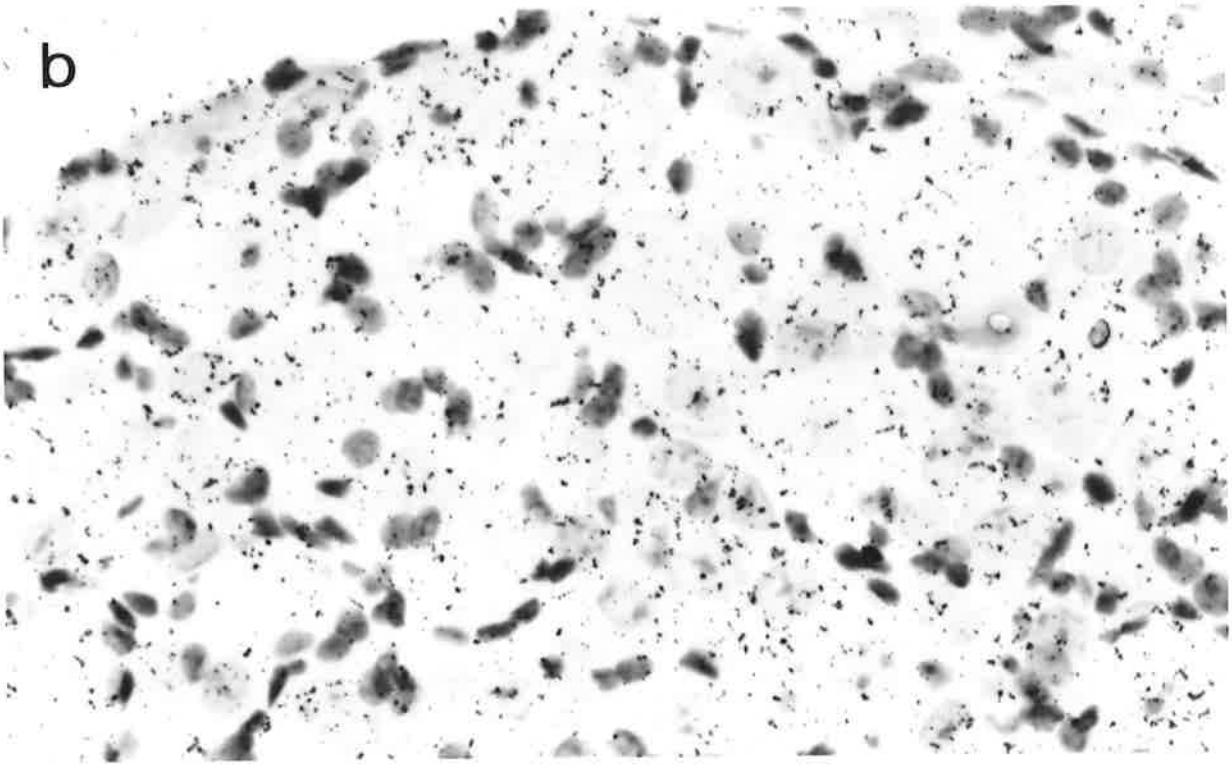

b

Figure 3.2. Specificity of LAT probe for HSV-infected tissues. Autoradiographic grains showing strong hybridization over a neuron (arrow) in a section of a thoracic ganglion (x400) taken from a BALB/c mouse 150d after infection (a). Clusters of grains were not observed over neurons in a ganglion (x400) taken from an uninfected mouse (b).


a


b

(i) Sections (5μ) of latently infected ganglia (150 days after infection) were digested with ribonuclease A prior to application of probe, which resulted in almost complete ablation of hybridization. Conversely, pretreatment of sections with DNAse did not appreciably alter the intensity of hybridization. It was concluded that pBS0 T7 transcripts detect RNA rather than DNA molecules in latently infected tissues.

(ii) Single stranded probes of the same sense as LATs (T3 transcripts of pBS0) were applied to sections of latently infected tissue (150 days after infection). No hybridization was detected, confirming the conclusions obtained from nuclease experiments.

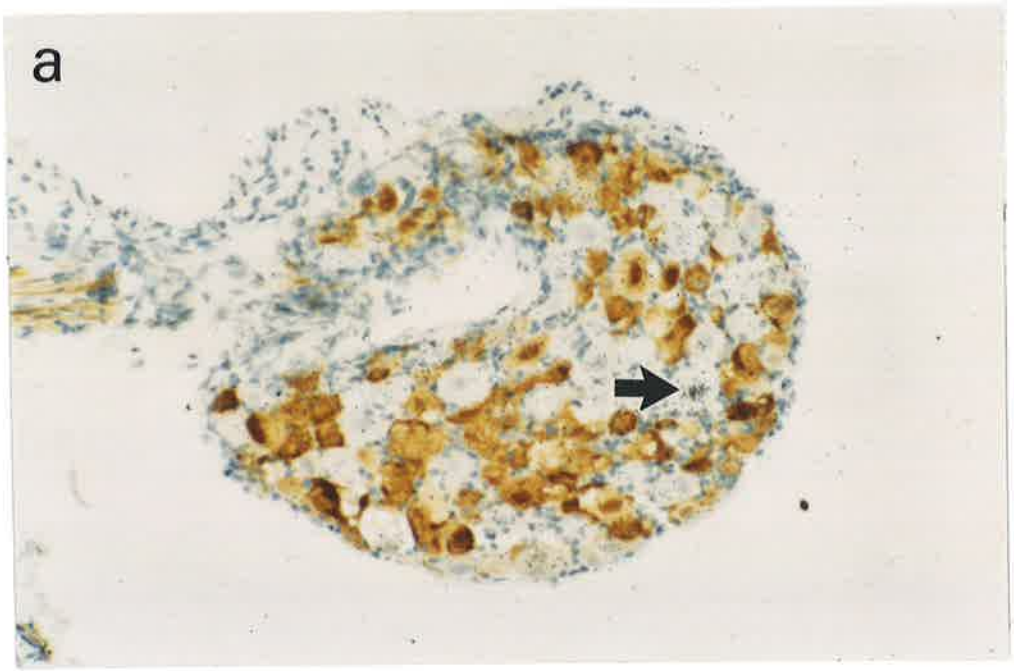
3.2 Dual labelling procedure

Simultaneous detection of nucleic acids and proteins requires: (i) preservation of nucleic acids and viral antigens (ii) two labelling systems and; (iii) preservation of tissue morphology (Brahic *et al*, 1984). Use of diaminobenzidine (DAB) as substrate for peroxidase during antigen detection produces a permanent, water and alcohol insoluble record of the presence of antigen, which resists subsequent ISH procedures (Brahic *et al*, 1984; Gowans *et al*, 1989).

A large number of slides (≥ 30) of pooled thoracic ganglia taken either from uninfected BALB/c or from acutely infected mice (days 4 and 5 after flank inoculation) were randomly selected for use in characterization of dual labelling. The number of individual ganglionic sections on each slide ranged from 10 through 21. Slides were subjected either to dual labelling or to ISH alone. Immunohistochemistry was with a polyclonal antiserum against HSV-infected cells and a LAT-specific probe was used in ISH. Neuronal profiles were randomly selected for enumeration of autoradiographic grains (Fig 3.4). Examination of immunohistochemically stained sections with or without subsequent ISH (Fig 3.3 a&b) showed that staining with DAB was unaffected by the ISH procedure. Grain counts over neuronal profiles in sections of uninfected tissue were the same for slides dual labelled or subjected to ISH alone (Fig 3.4). LAT^+ neurons were detected in sections of infected tissue subjected to dual labelling or to ISH alone (Fig 3.4). Immunohistochemical staining did not prevent access of radio-labelled RNA probes from reaching their target (Fig 5.1c).

It was concluded that (i) sensitivity of detection of HSV antigens suffers no apparent loss through a subsequent ISH procedure, (ii) that the background levels of autoradiographic grains over dual labelled sections and sections treated with ISH alone are similar, and (iii) that HSV nucleic acids can be detected in tissues that have been subjected to immunohistochemistry.

Figure 3.3. Similar intensity of immunohistochemical staining in sections of a spinal ganglion, taken from a BALB/c mouse 5 days after infection, subjected to dual labelling (a, x125) or to immunohistochemistry alone (b, x100). *In situ* hybridization was with LAT-specific probe and immunohistochemistry utilized a polyclonal antiserum directed against HSV infected cells. The arrow in panel (a) points to a LAT^+ Ag^- neuron.


a

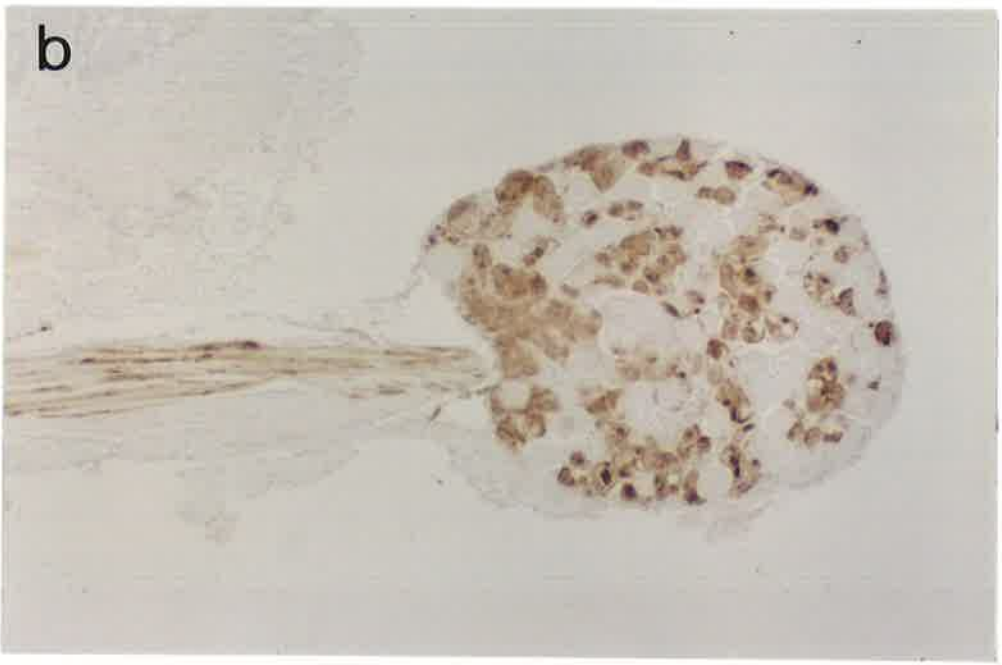
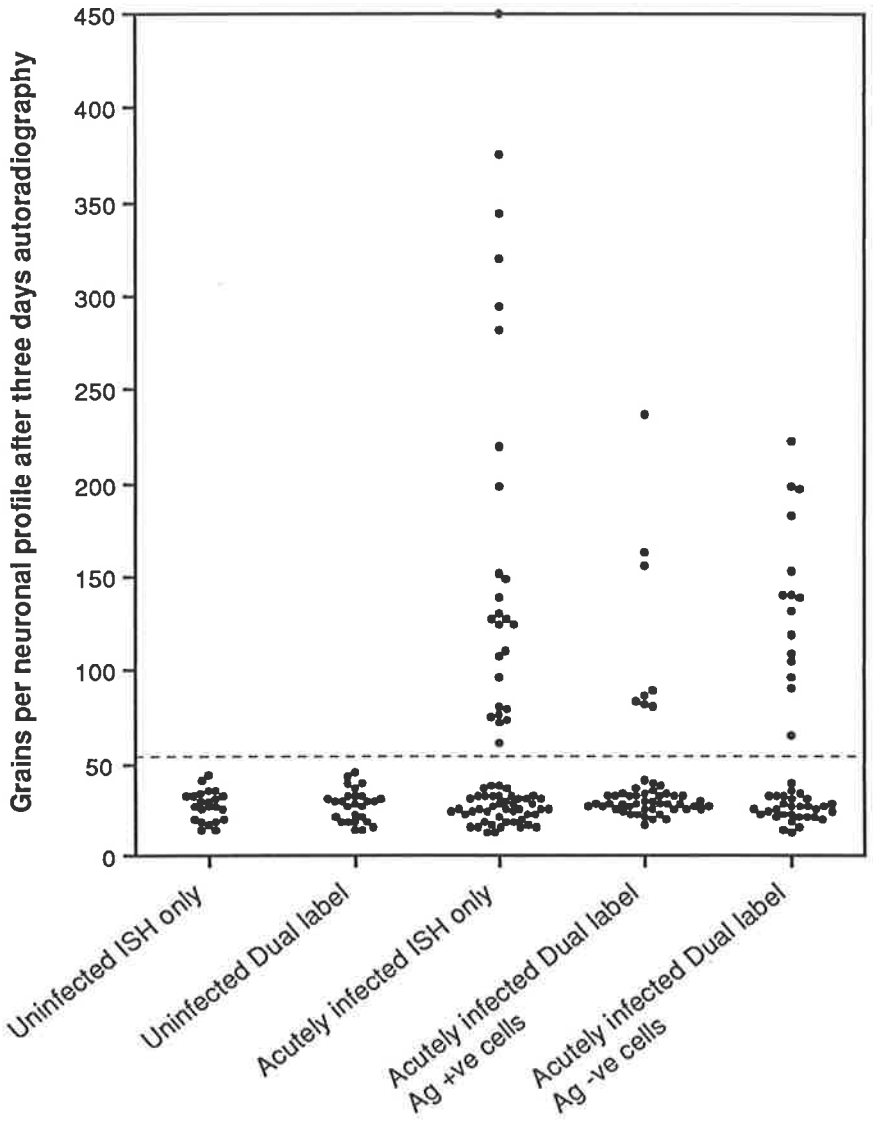

b

Figure 3.4. Distribution of autoradiographic grain counts over neuronal profiles in ganglionic sections subjected to dual labelling or ISH alone. Ganglionic sections derived from uninfected or acutely infected mice (days 4 and 5 after inoculation) were hybridized to a LAT-specific probe, with or without preceding immunohistochemistry using polyclonal antiserum directed against HSV-infected cells. Neuronal profiles were randomly selected for enumeration of grains. Each dot represents the grain count over a neuronal profile. Dashed line is 3 SD above the mean grain count per neuronal profile on sections of uninfected tissue subjected to ISH alone.


Grains per neuronal profile after three days autoradiography
450
400
350
300
250
200
150
100
50
0
Uninfected ISH only
Uninfected Dual label
Acutely infected ISH only
Acutely infected Dual label
Ag +ve cells
Acutely infected Dual label
Ag -ve cells

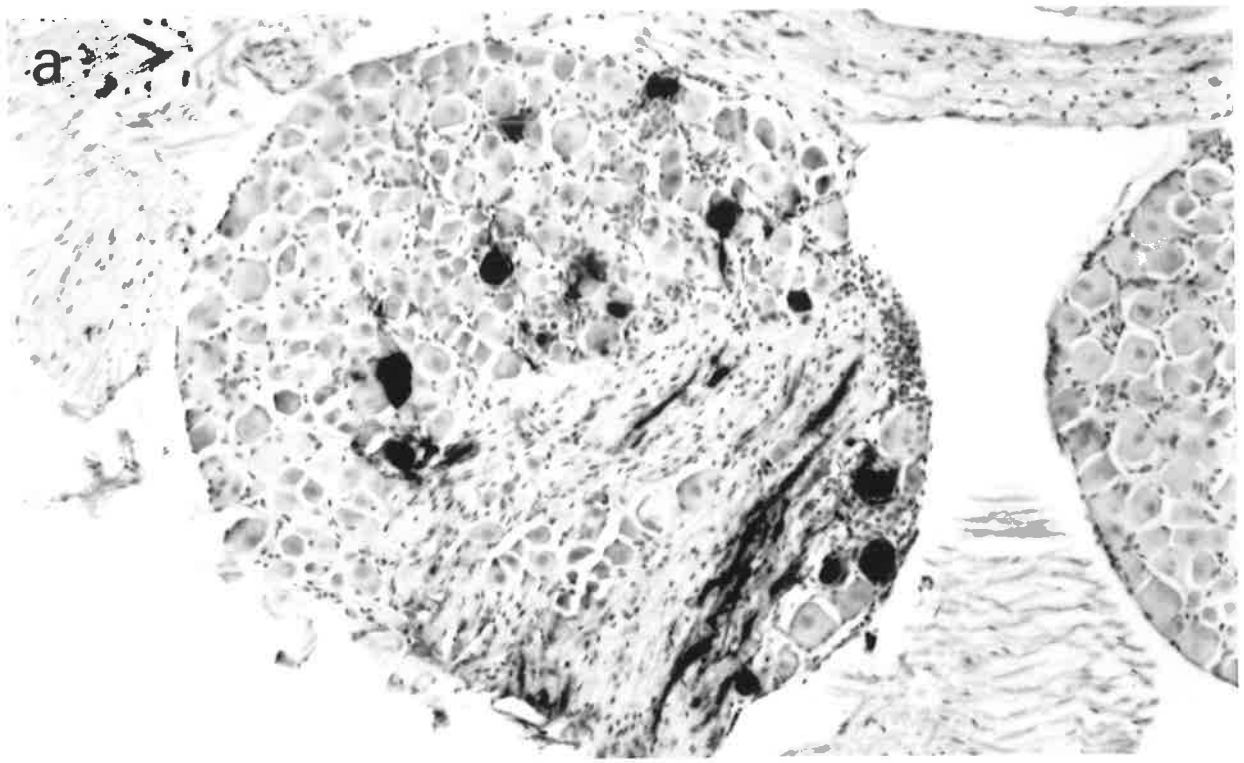
4. MOLECULAR PATHWAYS OF PRODUCTIVE AND LATENT INFECTION FOLLOWING INFECTION WITH A VIRULENT STRAIN OF HSV-1

4.1 Acute and latent infection in the peripheral nervous systems of C57BL10 and BALB/c mice

The proportion of ganglionic cells expressing viral antigens was determined daily throughout the entire course of acute infection in C57BL10 and BALB/c mice by immunohistochemical analysis of dorsal root ganglia (T8-T13 pooled) removed from groups of 10 animals from the 2nd-7th day after inoculation (d 0) of virus into the skin. Ganglia were removed from additional groups of mice after 120 days (C57BL10) or 150 days (BALB/c) and studied for the presence of LATs.

In C57BL10 mice, infection was detected first on d4 (one neuron only), peaked sharply on d5 (2.9% of neurons examined) and then cleared rapidly (Table 4.1). Viral antigens were largely confined to (i) neurons in ganglionic paranchyma and (ii) axons and Schwann cells in nerve fibres (Fig 4.1 a&b). Antigen positive neurons were notably clustered into a minority of the ganglia visualised in each section. After 120d LATs were detected in 5.1% of neurons (Fig 4.2a) which were scattered widely, such that the majority of ganglia examined (68%) showed evidence of latency.

Figure 4.1. Visualization of infected cells by immunohistochemistry during acute ganglionic infection of C57BL10 mice. HSV antigens (black) were largely confined to neurons and nerve fibres in ganglionic parenchyma (a, x125) and axons and Schwann cells in nerve fibres (b, x625).


a

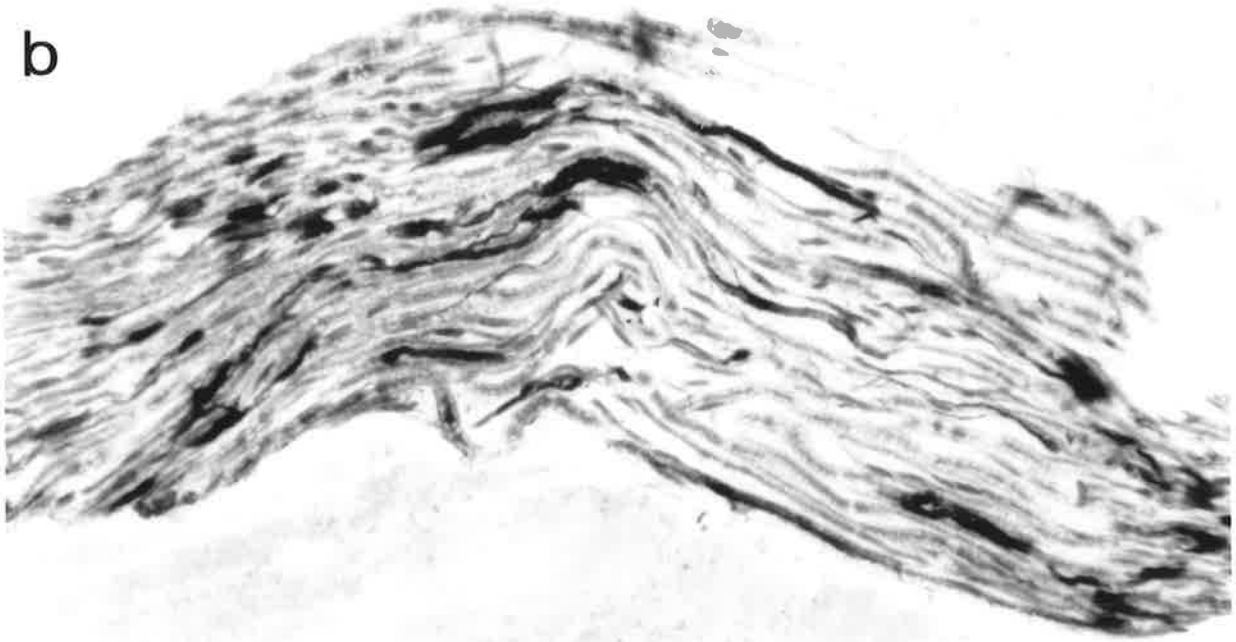
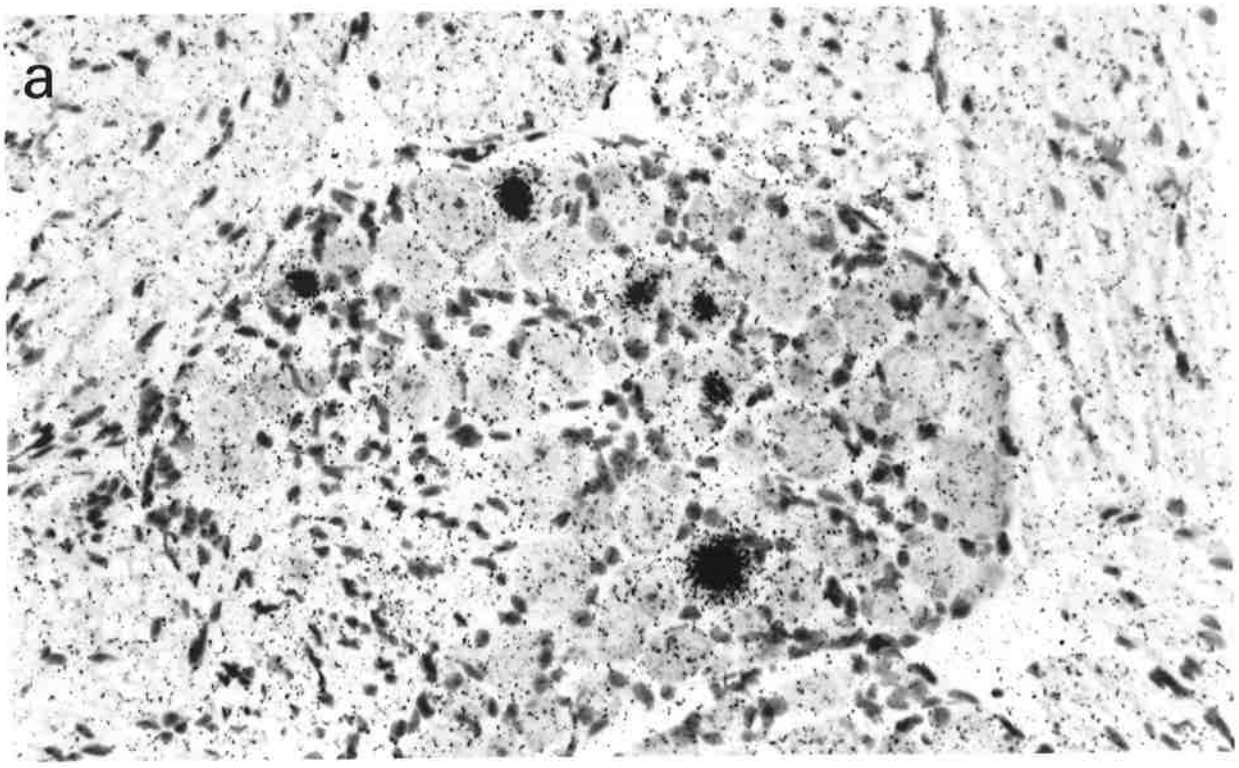

b

Figure 4.2. Detection of LATs by *in situ* hybridization to sections of thoracic ganglia taken from (a) C57BL10 mice 120d after infection (x250) and (b) BALB/c mice 150d after infection (x160). Autoradiographic grains were clustered over neuronal nuclei.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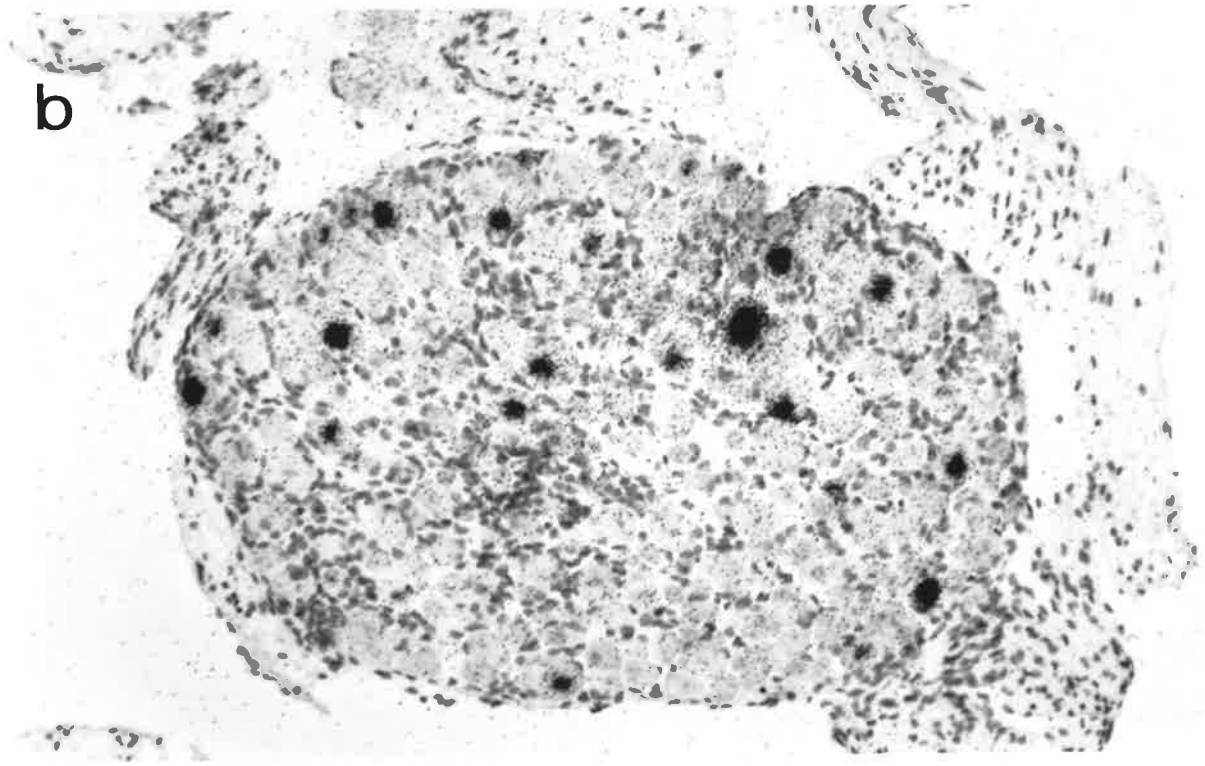

b

In BALB/c mice (Table 4.2), Ag^+ neurons were detected on d4 in a higher proportion of pooled ganglia (T8-T13) than in C57BL10 animals (20% vs 2.3% respectively). Similarly, a higher proportion (44%) of BALB/c ganglia were Ag^+ at the peak of infection (d5) compared with C57BL10. After 150 days, LATs were detected in 3.9% of neurons and the majority of BALB/c ganglia examined (67%) showed evidence of latency (Fig 4.2b).

Speed of recovery in C57BL10 mice was examined in more detail. Thoracic ganglia (T8-T13) were removed from groups of 3 C57BL10 mice 5, 6 and 7 days after infection and tested for the presence of infectious virus by plaque assay. In this experiment infection had reached a steady state by day 5, recovery began on day 6 and infectious virus was undetectable by day 7 (Fig 4.3a). It was concluded that infectious HSV is rapidly removed from the PNS of C57BL10 mice between approximately 144 and 168 hours after infection. Detailed analysis of the recovery phase was based on a series of 'snapshots' generated from groups of 10 mice, killed at 8 hourly intervals between 120 and 152 hours after infection. At each timepoint dorsal root ganglia (T8-T13) and the proximal portion of the spinal nerve trunk ipsilateral to the inoculation site were removed, pooled and studied for the presence of viral antigens and thymidine kinase mRNA. Infection peaked 128-136 hours after flank inoculation judged by the proportion of randomly selected ganglionic sections containing antigen positive profiles (Fig 4.3b) or by the average number of antigen positive neuronal profiles in each ganglion (not shown). Eight hours

Figure 4.3. Analysis of recovery from acute ganglionic infection in C57BL10 mice. Thoracic ganglia (T8-T13) were removed from groups of 3 C57BL10 mice 5, 6 and 7 days after infection and tested for the presence of infectious virus by plaque assay (a). At 8 hourly intervals between 120 and 152 hours after infection, spinal ganglia (T8-T13) were removed from groups of 10 mice, pooled, and randomly selected sections were studied for the presence of viral antigens (b) and viral thymidine kinase mRNA (c). Clearance of infectious virus and viral materials was precipitous.

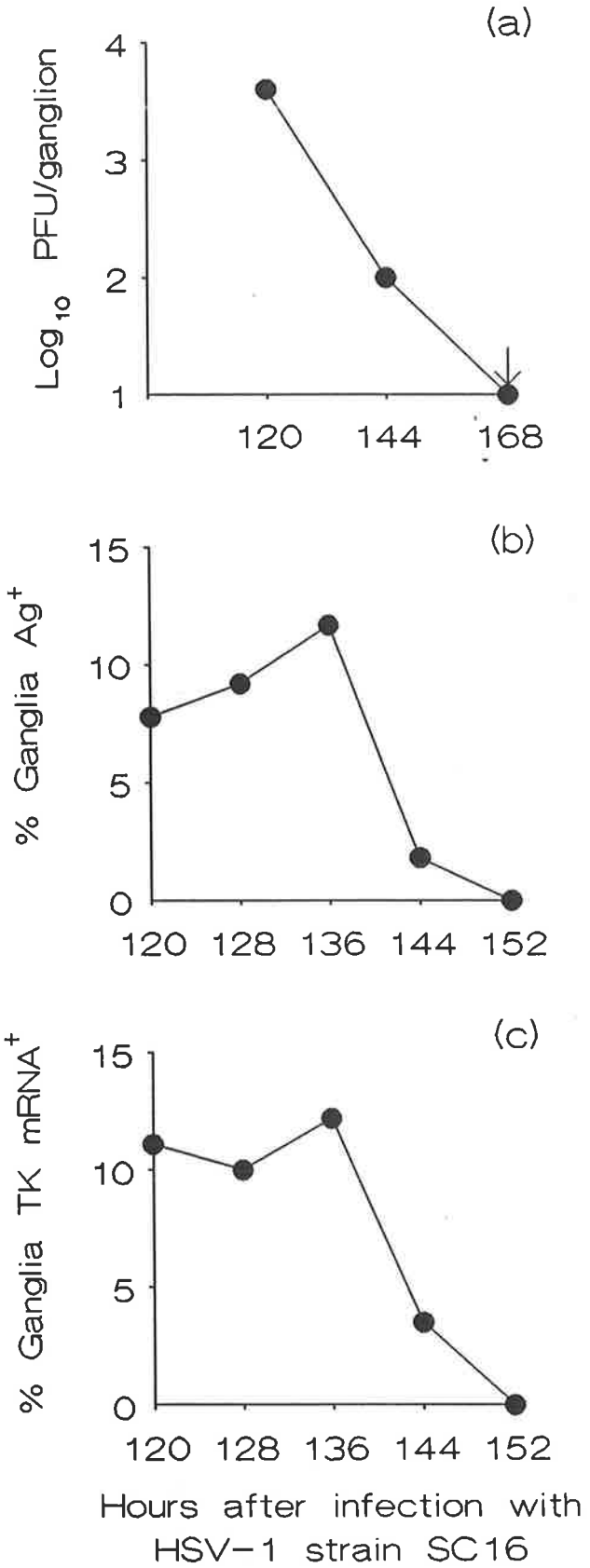

(a)
Log10 PFU/ganglion
4
3
2
1
120
144
168
(b)
% Ganglia Ag+
15
10
5
0
120
128
136
144
152
(c)
% Ganglia TK mRNA+
15
10
5
0
120
128
136
144
152
Hours after infection with
HSV-1 strain SC16

TABLE 4.1. EXTENT OF PRODUCTIVE AND LATENT INFECTION IN C57BL10 MICE

DAY AFTER INFECTION	NO. OF NEURONS EXAMINED	NO. OF NEURONS Ag + (%)	NO. OF GANGLIA EXAMINED	NO. OF GANGLIA Ag + (%)	NO. OF NEURONS LAT + (%)	NO. OF GANGLIA LAT + (%)
2	4692	0	34	0	N.T.[1]	N.T.
3	4692	0	34	0	N.T.	N.T.
4	5934	1 (0.02)	43	1 (2.3)	N.T.	N.T.
5	9936	285 (2.9)	72	20 (28)	N.T.	N.T.
6	6348	72 (1.1)	46	9 (20)	N.T.	N.T.
7	3174	4 (0.13)	23	3 (13)	N.T.	N.T.
120	17940	0	130	0	913 (5.1)	88 (68)

[1] N.T. = Not tested

TABLE 4.2. EXTENT OF PRODUCTIVE AND LATENT INFECTION IN BALB/C MICE

DAY AFTER INFECTION	NO. OF NEURONS EXAMINED	NO. OF NEURONS Ag + (%)	NO. OF GANGLIA EXAMINED	NO. OF GANGLIA Ag + (%)	NO. OF NEURONS LAT + (%)	NO. OF GANGLIA LAT + (%)
2	5106	0	37	0	NT	NT[1]
3	6624	0	48	0	NT	NT
4	7590	92 (1.2)	55	11 (20)	NT	NT
5	8142	680 (8.3)	59	26 (44)	NT	NT
6	11454	226 (2.0)	83	16 (19.3)	NT	NT
7	8832	54 (0.6)	64	12 (18.8)	NT	NT
150	10764	0	78	0	421 (3.9)	52 (67)

[1]NT = not tested

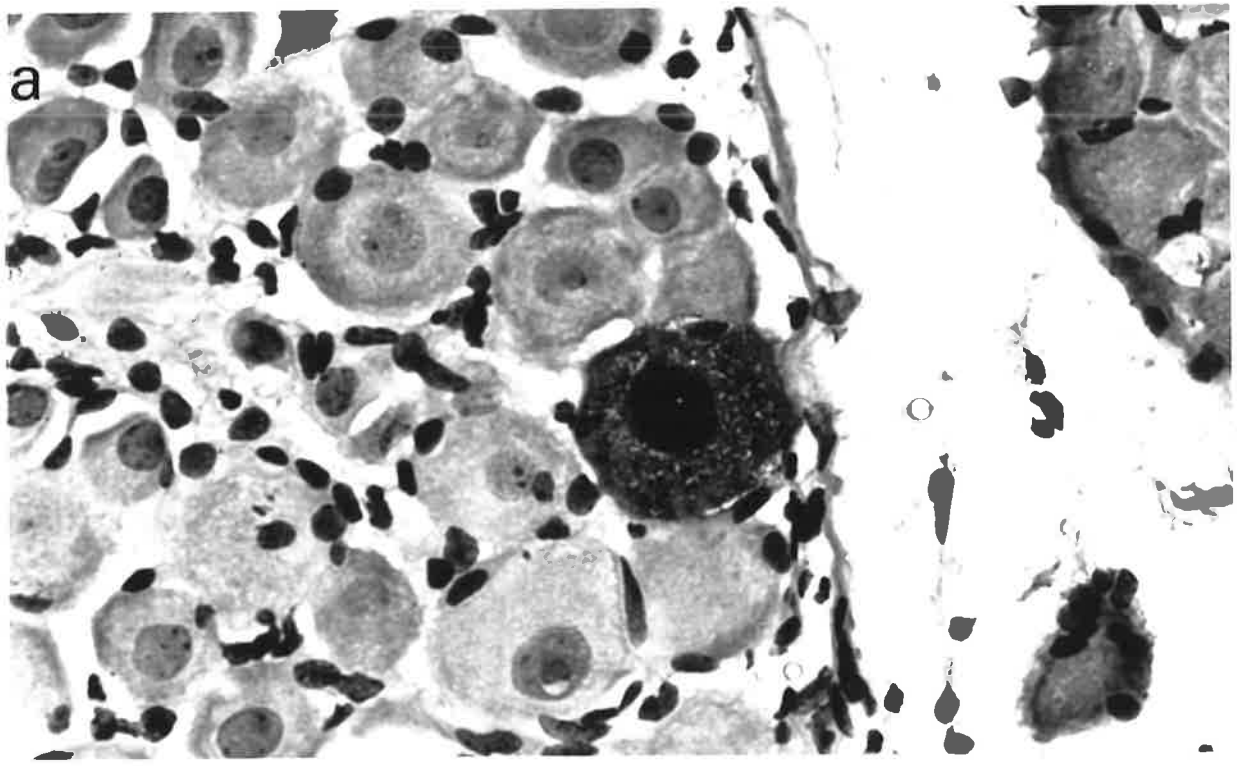
after the peak of infection very few ganglionic sections contained detectable viral antigen and after a further eight hours infected cells could not be detected. Presence of HSV thymidine kinase mRNA correlated closely with the presence of viral antigen (Fig 4.3c). It was concluded that (i) acute ganglionic infection in the C57BL10 mouse is abruptly terminated over an 8-16 hour period and (ii) viral gene products are very quickly removed from ganglia. Viral antigens and thymidine kinase mRNA were detected in primary sensory neurons but not in the much smaller and more numerous capsular cells (Fig 4.4a) which surround each neuron. Many antigen positive neurons showed no overt disturbance of architecture; characteristic changes induced by replication of HSV in cell culture, such as nucleolar and nuclear displacement and degeneration and ballooning of cells were not consistently observed in ganglionic neurons in the model system described here. Cell fusion was never seen, even in heavily infected tissue.

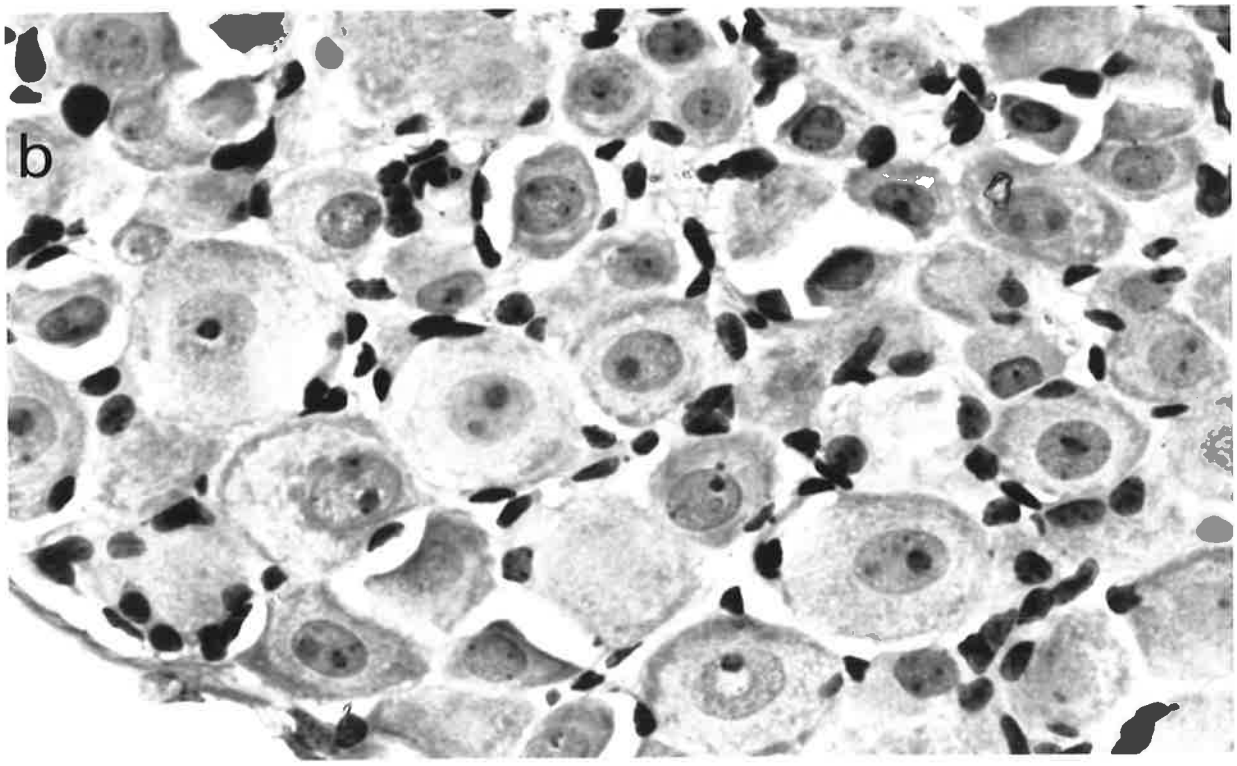
4.2 Anatomical distribution (T6-L1) of viral gene expression in C57BL10 mice, during establishment phase

The discrepancy, in both mouse strains, between the proportion of latently infected ganglia and the proportion expressing viral antigens during the establishment phase suggests that the antigen expressing cells are not essential precursors of the neurons harbouring long term transcriptionally active viral genomes. A more detailed analysis of viral activity at specific anatomical locations was undertaken. C57BL10 mice were selected for this experiment

Figure 4.4. (a) Morphologically preserved antigen-positive neuron (x625) present during recovery from acute ganglionic infection in C57BL10 mouse, demonstrated immunohistochemically using polyclonal antiserum directed against HSV-infected cells.

(b) Normal appearance of primary sensory neurons (x625): these cells are readily identifiable by their large size, characteristic nucleic acid-rich cytoplasm, and ovate nucleus with one or more prominent nucleoli. Surrounding capsular cells have little or no visible cytoplasm.

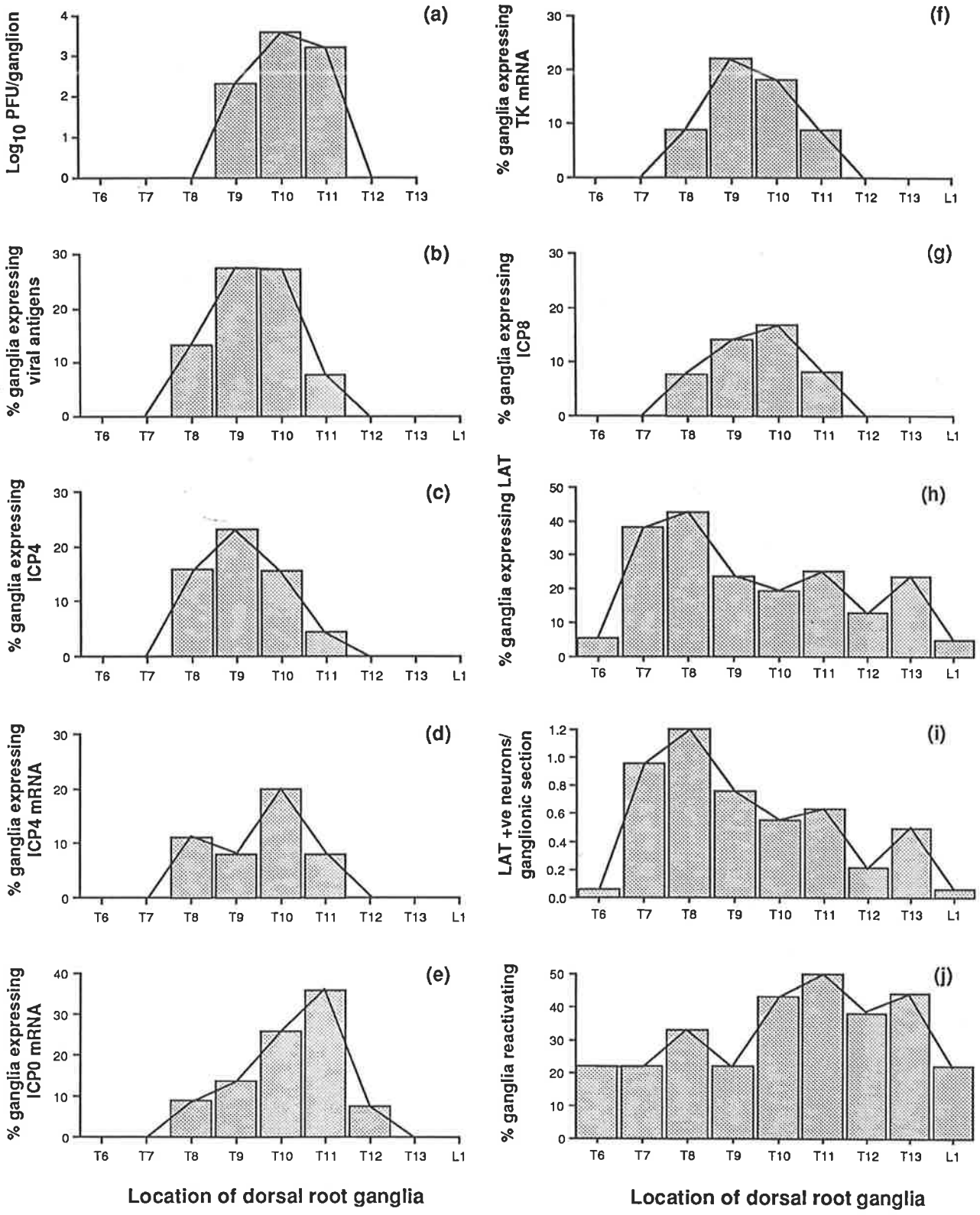

a


b

based on the greater discrepancy between the proportion of antigen positive and LAT^+ neurons detected in pooled samples, compared with BALB/c animals (see tables 4.1 and 4.2). Mice were infected within the region of the 10th thoracic dermatome, and 10 animals were killed at 24h intervals between d4 and d7 in order to study ipsilateral dorsal root ganglia between T6 and L1 for evidence of viral activity (by immunohistochemistry for selected gene products or ISH for mRNAs). Fixed tissues were pooled with respect to vertebral level prior to embedding in paraffin blocks. Forty mice were allowed to survive until d45, at which time 30 were used to study the location of LATs and ten were used for classical explant reactivation studies on individual ganglia. At the peak of infection (d5) ganglia were removed from an additional 3 mice and tested for the presence of infectious virus which was found only at the 9th, 10th and 11th thoracic levels, peaking at T10 (Fig 4.5a). Immunohistochemical analysis using a polyclonal antiserum directed against a wide variety of infected cell polypeptides showed that the distribution of antigen positive neurons was also restricted (Fig 4.5b). Despite examination of multiple randomly selected sections (with typically 20 ganglia per section) HSV antigen expression at T6, T7 or T12-L1, was not detected.

Specific evidence of expression of the immediate early gene encoding ICP4 was sought, because initiation of the HSV replicative cycle in cell culture is dependent on this gene. With apparently similar sensitivity both ICP4 and its mRNA were detected, by immunohistochemistry and ISH respectively, at sites

Figure 4.5. Analysis of acute and latent infection in spinal ganglia (T6-L1) after inoculation of virus at T10. Ganglia from 3 mice were tested for the presence of infectious virus at the peak of infection (a). Ganglia from 40 mice representing the whole of the acute phase of infection (d4-d7) were analysed for expression of proteins detected by broad spectrum anti-HSV antibodies (b), expression of ICP4 (c), ICP4 mRNA (d), ICP0 mRNA (e) TK mRNA (f), and ICP8 (g). Ganglia from a further 40 mice were removed 45d after infection and analysed for the presence of LATs by *in situ* hybridization (h and i) or reactivation by explant culture (j). Evidence of viral gene expression was confined to the T8-T12 region, whereas LATs and reactivation were widespread.


(a)
Log10 PFU/ganglion
(b)
% ganglia expressing viral antigens
(c)
% ganglia expressing ICP4
(d)
% ganglia expressing ICP4 mRNA
(e)
% ganglia expressing ICP0 mRNA
(f)
% ganglia expressing TK mRNA
(g)
% ganglia expressing ICP8
(h)
% ganglia expressing LAT
(i)
LAT +ve neurons/ ganglionic section
(j)
% ganglia reactivating
T6 T7 T8 T9 T10 T11 T12 T13 L1
Location of dorsal root ganglia
Location of dorsal root ganglia

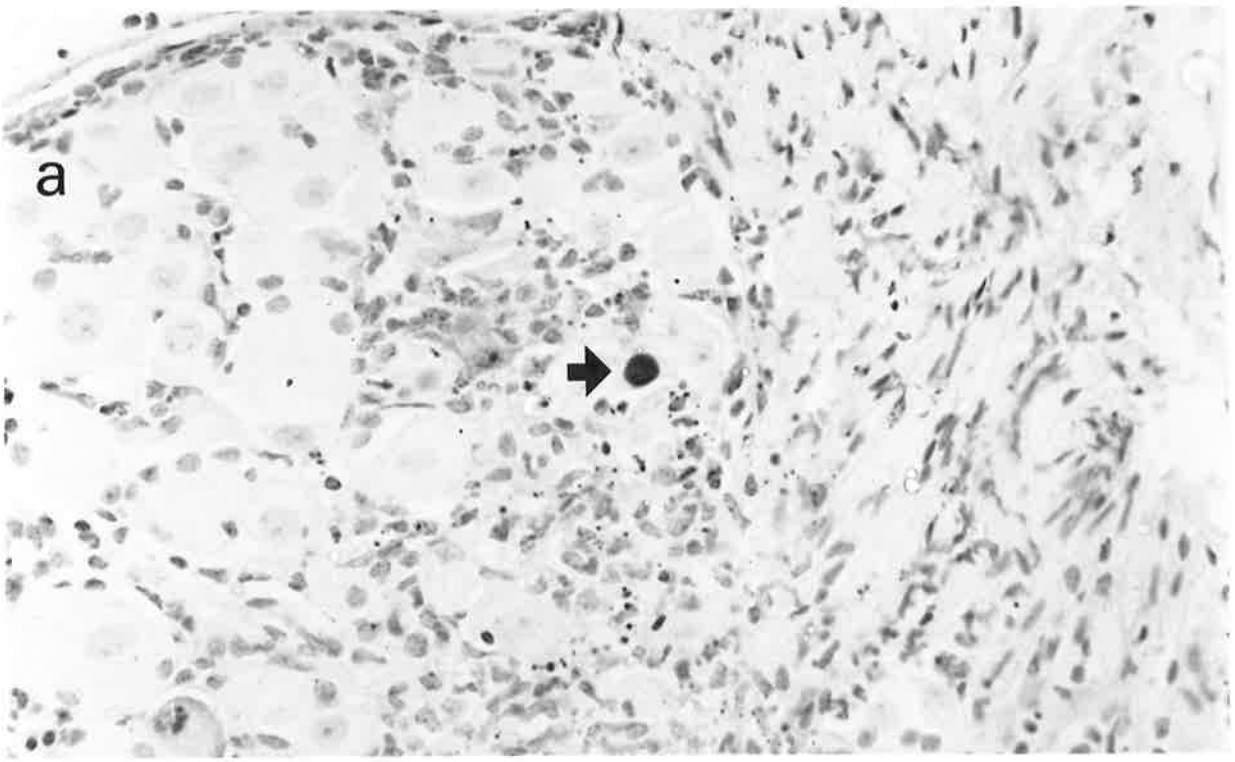
associated with productive infection i.e. within the limits of T8-T11 (Fig 4.5 c&d; Fig 4.6a). Messenger RNA of ICP0 (Fig 4.7a), which is partially complementary to the most abundant LATs was detected with slightly greater frequency than ICP4 mRNA (Fig 4.7b) but in a similar pattern, extending caudally to T12 (Fig 4.5e). Expression of TK mRNA was confined between the limits T8-T11 (Fig 4.5f).

Replication of HSV DNA is thought to require the presence of the major DNA binding protein ICP8, an early viral gene product. This protein could be readily detected immunohistochemically in the nuclei of infected neurons (Fig 4.6b) but again its presence was restricted to ganglia between T8-T11 (Fig 4.5g).

To corroborate the data collected from pooled ganglia, the distribution of viral gene expression within the PNS was studied using coronal sections of spinal columns (Fig 4.8) taken from groups of three infected mice killed daily between 2 and 7 days after infection. In this experiment viral antigens were detected (using a polyclonal antiserum against HSV infected cells) five days after infection (Fig 4.9) in segments T10, T11 and T12 and again six days after infection in segment T11 only. No evidence of infection was seen before day 5 or after day 6.

It was concluded that after inoculation of virus into the flank skin of C57BL10

Figure 4.6. Immunohistochemical detection of specific viral proteins during acute ganglionic infection of C57BL10 mice. Staining for ICP4 (a, arrowed) and ICP8 (b) was restricted to neuronal nuclei (x400).


a

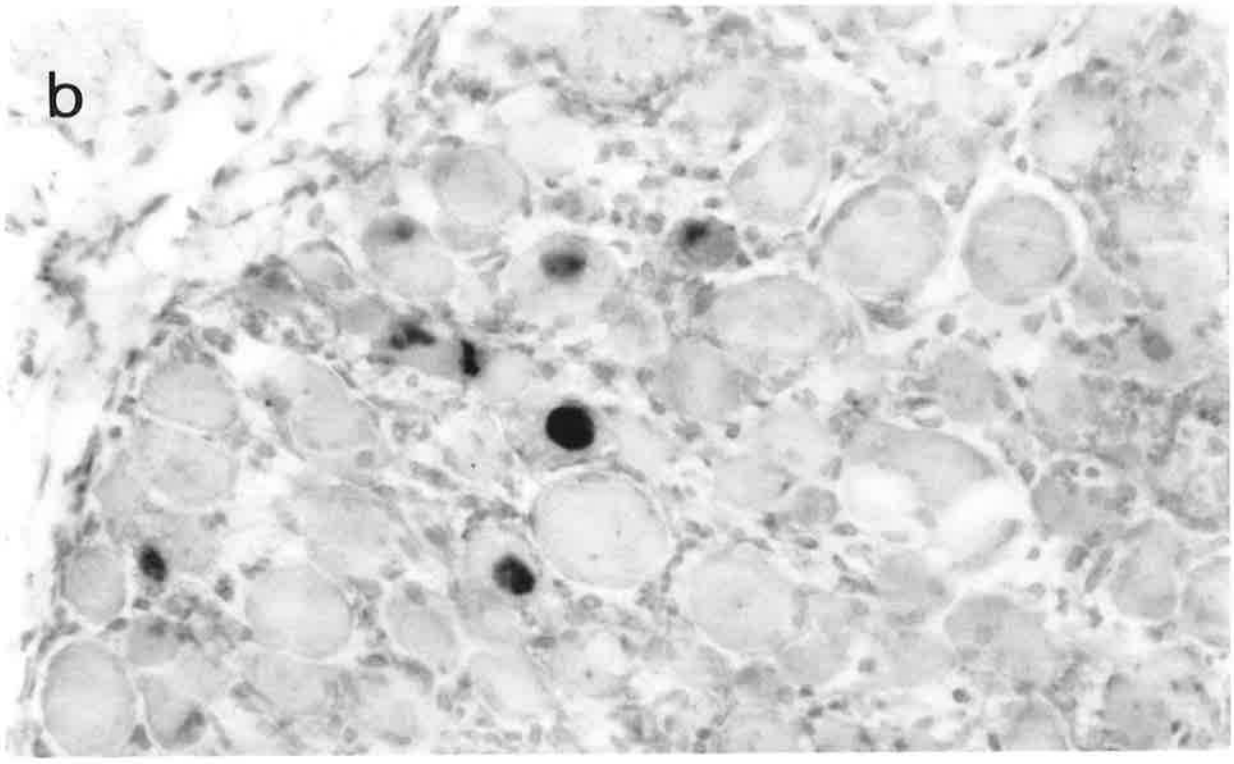
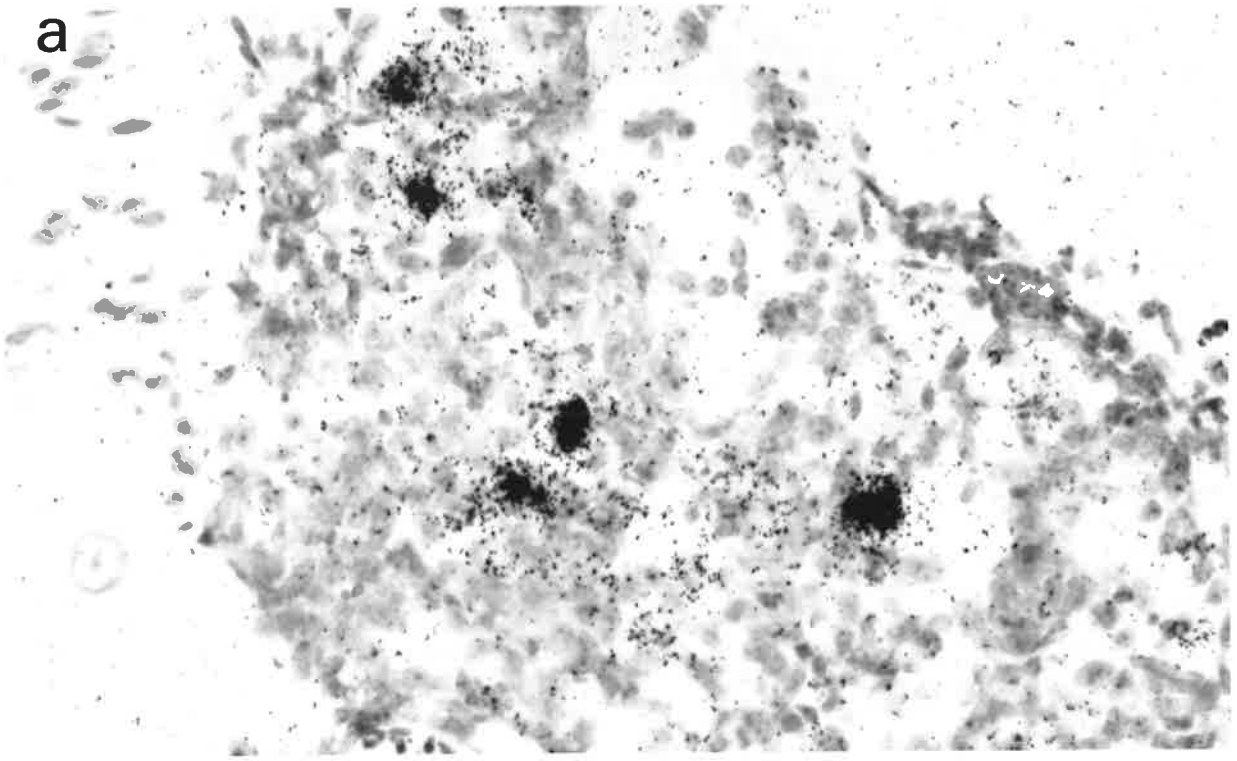

b

Figure 4.7. Photomicrographs (x250) showing detection by ISH of specific HSV-1 messenger RNAs during acute ganglionic infection of C57BL10 mice. Autoradiographic grains show hybridization of probes specific for messenger RNA of (a) ICP0 and (b) viral thymidine kinase.


a

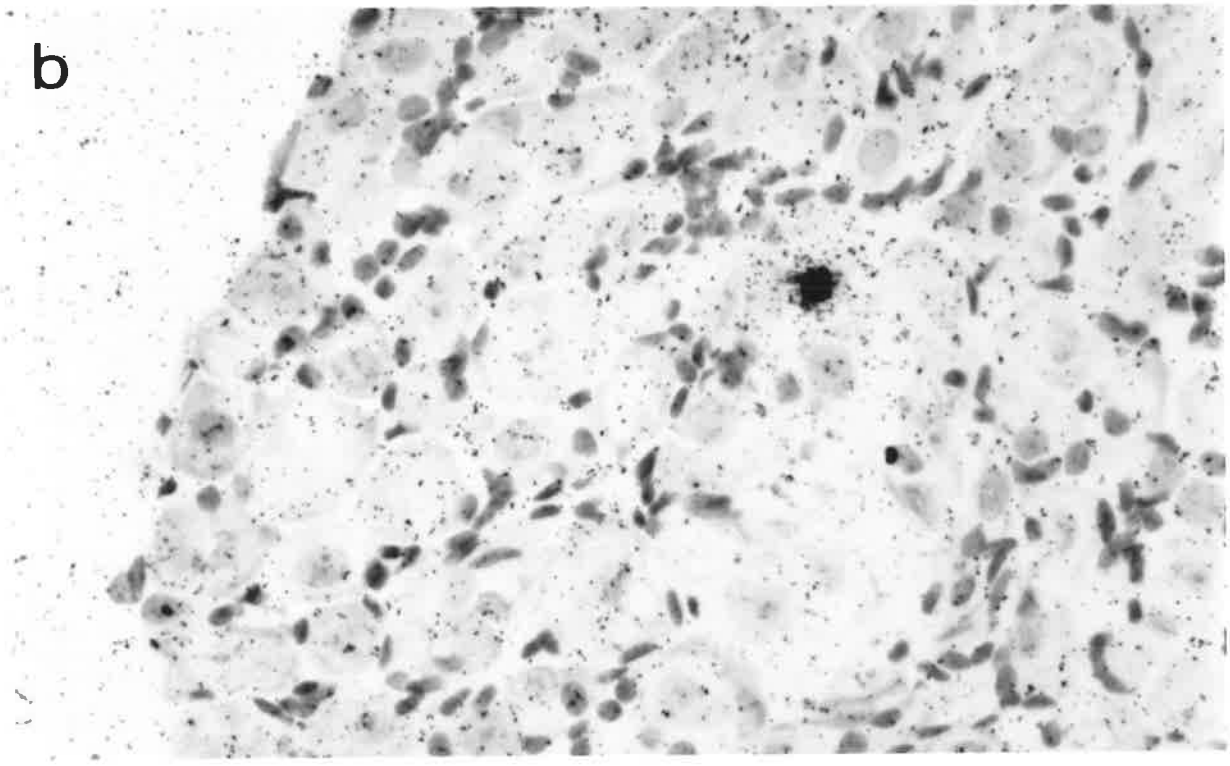

b

Figure 4.8. Coronal section of the lower thoracic section of the spinal column of a mouse, illustrating spinal cord, dorsal nerve roots (arrowheads), dorsal root ganglia (arrows) vertebrae (v) and ribs (R) (Magnification: x20).

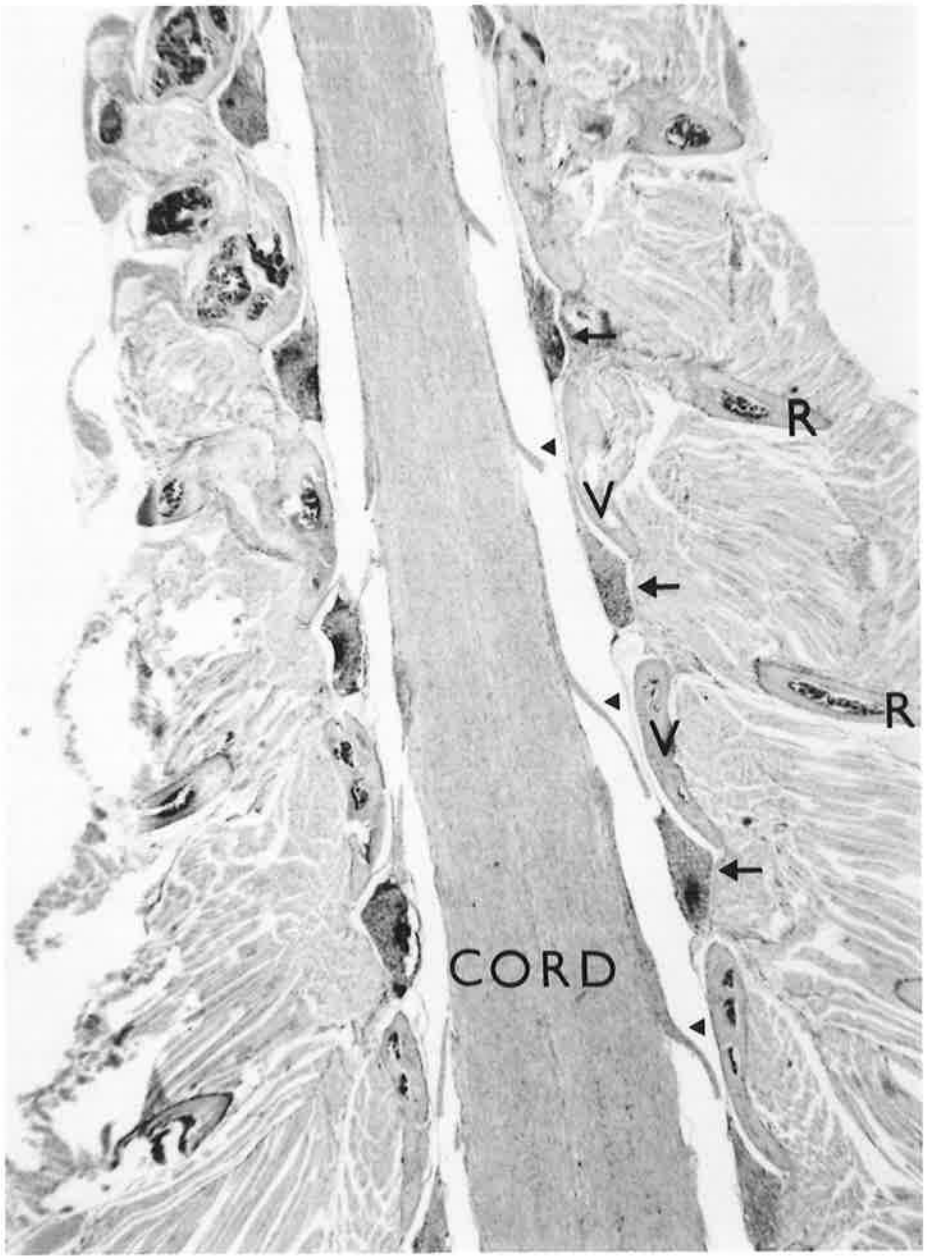
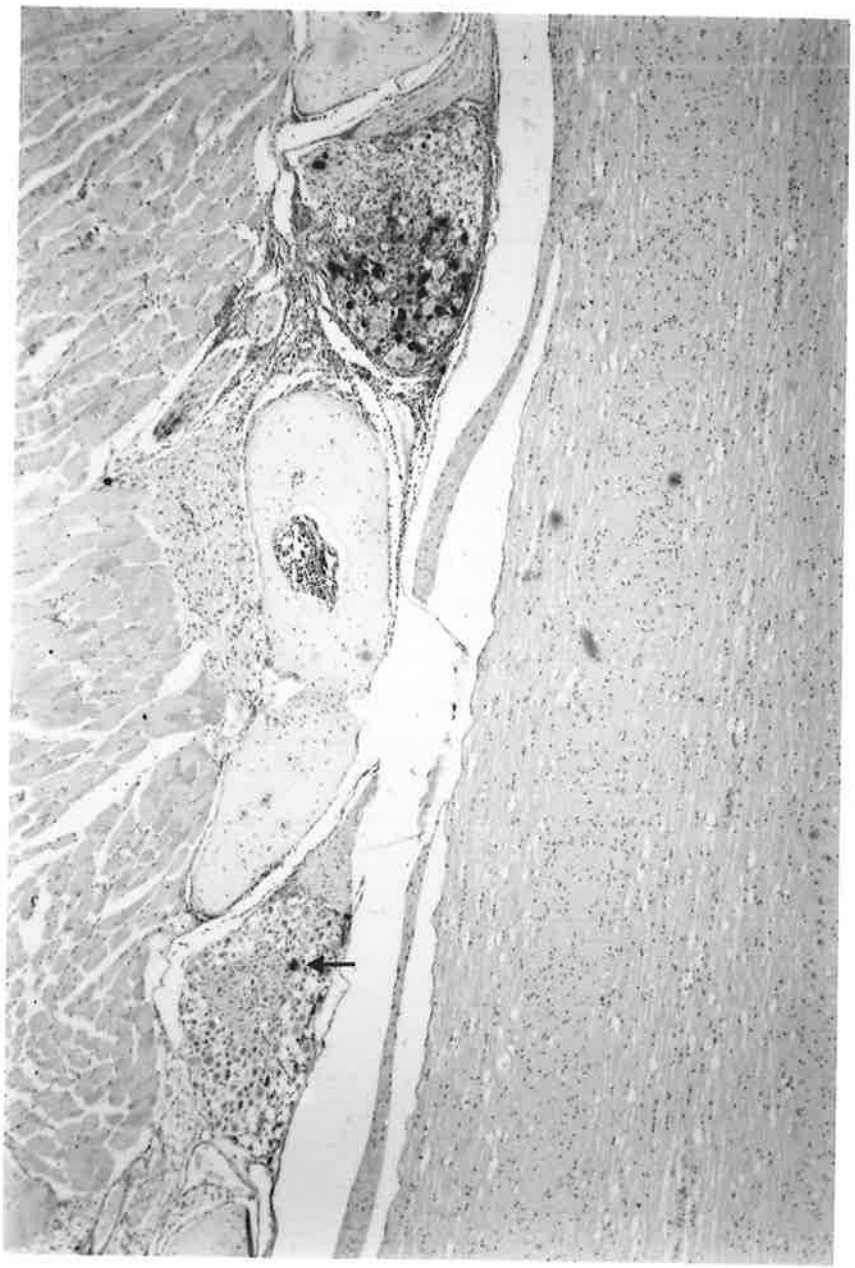

R
V
R
V
CORD

Figure 4.9. Immunohistochemical detection of viral antigens in a coronal section (x50) of the spine of a C57BL10 mouse 5 days after flank inoculation. Numerous antigen-positive neurons (darkly staining cells) are visible in the 11th thoracic ganglion, seen in cross section at the top of the photo. Arrow points to a single positive staining neuron in the 12th thoracic ganglion.



mice productive infection and detectable expression of ICP4, ICP0, TK and ICP8 and other immunogenic infected cell polypeptides during acute infection (corresponding to the phase in which latency is established), are restricted to ganglia between T8 and T12.

4.3 Anatomical distribution (T6-L1) of latency

Latent infection was widespread such that in 30 mice killed for histological studies on d45, neurons containing LATs were found at all sites examined (Fig 4.5 h and i). Using the classic approach of explant culture, virus could be reactivated from all levels tested (T6-L1) (Fig 4.5j); particular attention is drawn to T7 and T13 where a substantial proportion of ganglia became latently infected with SC16 despite absence of detectable gene expression (including ICP4 and ICP0) and infectious virus at these levels during the establishment phase. It was concluded that the molecular pathways of productive and latent infection with a virulent strain of HSV-1 can diverge from a very early stage.

4.4 Discussion

The studies reported in this chapter were made possible by the unique nature of the experimental model used. Segmental innervation of the vertebrate trunk, in the form of overlapping dermatomes provided an ideal system in which to monitor the spread of HSV from the skin to sensory ganglia that directly innervate the site of inoculation and from there to ganglia that innervate neighbouring parts of the flank. Mice were infected in the tenth thoracic

dermatome, which is overlapped rostrally by T9 and caudally by T11. It is recognised also that some neurons situated in the eighth and twelfth thoracic ganglia might also send nerve fibres to the infected skin. Spread of virus from the skin directly to ganglia outside these limits is very unlikely. During the acute phase of infection the distribution of infectious virus and cells expressing viral genes correlated closely with the expected pattern of innervation of the inoculation site, confirming the power of HSV as a neurological tracer (Ugolini *et al*, 1987, 1989). Within ganglia infection was largely restricted to neurons and indeed viral antigen was rarely detected in the capsular cells that surround each neuron. The widely scattered distribution of infected cells and the absence of syncytium formation in ganglia support the conclusions of others that HSV does not spread directly from neuronal somata to other neurons or to surrounding support cells (Cook and Stevens, 1973). Neural spread of HSV more likely involves the central processes of sensory neurons as a result of their communication within the spinal cord (Simmons and Nash, 1987).

Rapid, uncontrolled spread of HSV throughout the nervous system is a lethal complication of the model system described when susceptible mouse strains (e.g. BALB/c) are used. C57BL10 mice did not entirely restrict the spread of virus but the outcome of infection in ganglia not directly innervating the inoculation site was establishment of latency, without apparent viral gene expression, rather than virus replication.

It is possible that, during the establishment of latency, viral proteins are produced in amounts not detected by either immunohistochemical procedures or ISH, or that an extremely transient burst of gene expression was overlooked. However, because expression of ICP4 (an immediate-early gene), ICP8 (an early gene regarded as essential for viral DNA replication) and structural genes is represented in the analysis, it can be concluded that if viral gene expression does occur, then from the earliest stages, it is quantitatively and/or qualitatively different from that associated with productive infection. By demonstrating that a virulent strain of HSV can establish latency without detectably initiating the pathway associated with productive infection, the experiments described here confirm and extend observations made with viral mutants but leave unresolved the issue of how a strikingly large amount of viral DNA remains in latently infected tissue after resolution of a natural infection. The bulk of this DNA may be a residuum of aborted productive infection, perhaps unrelated to the transcriptionally active viral genomes in LAT positive neurons. This hypothesis can be tested using the model system described here, by determining the number of viral genomes present at each ganglionic level between T6 and L1 in latently infected animals.

Although generally within the same dermatome, the exact sites of primary and recrudescent herpes in humans are often different. For example primary gingivostomatitis can recur periodically as cold sores (Whitley, 1990) and herpetic whitlow in health care workers frequently recurs some distance from

the point of initial infection (Gill, 1990). An extension of this phenomenon is perhaps seen in the model described here, because latency was clearly detected in neurons that innervate regions of the flank distant from the inoculation site.

5. DUAL LABELLING STUDIES

5.1 Synchronous detection of viral antigens and latency associated transcripts in acutely infected ganglia

Results in the previous chapter suggested that antigen-expressing cells are not essential precursors of latently infected neurons. To test this hypothesis dual labelling studies were carried out to examine spinal ganglia through the course of the acute infection for the presence of latently infected neurons.

For the purpose of this study latently infected neurons were defined as those cells containing LATs in the absence of detectable viral proteins (Ag^-, LAT^+). If there is no precursor relationship between Ag^+ and Ag^-, LAT^+ cells, then, given sufficient sensitivity of the ISH procedure, both would be detectable in the PNS in the earliest stages of ganglionic infection.

Female BALB/c mice were flank-inoculated with HSV-1 strain SC16 and on days 2 to 5 and on day 45, left sensory dorsal root ganglia (T8-T13) were removed from groups of 10 mice, pooled, fixed in PLP, paraffin-embedded and sections (5 μm) were collected onto slides. Dual labelling (immunohistochemistry followed by ISH) was carried out on a large number of randomly selected ganglionic sections (ranging from 180 on day 2 down to 47 on day 5) and Ag^+ LAT^-, Ag^+ LAT^+ and Ag^- LAT^+ neuronal profiles were counted.

On day 2 neither viral antigens nor LATs were detected (Table 5.1). On day 3, Ag^+ LAT^- and Ag^- LAT^+ (Fig 5.1a) neurons were observed, occasionally in adjacent cells (Fig 5.1b) although Ag and LAT signals were not mutually exclusive. Approximately 14% of Ag^+ cells also contained LATs (Fig 5.1c). All three types of infected cell (Ag^+ LAT^+, Ag^+ LAT^-, Ag^- LAT^+) increased in number during the course of acute infection. The proportion of neurons containing LATs increased from 1.5% on day 5 to 3.6% on day 45.

The intensity of LAT specific autoradiographic signals on days 3 to 5 (typified in Figs 5.1 a&b) was at least an order of magnitude less than that typically observed in latently infected neurons 45 days after infection. On the basis of grain counts over infected neurons it is estimated that on days 3-5 LAT^+ Ag^- neurons each contain up to 10^3 viral transcripts in contrast to approximately 10^4 copies of LAT commonly detected on day 45 (Fig 5.2).

5.2 Discussion

This study relied on a series of snapshots of events taken at daily intervals, because it is not possible to follow individual neurons through a time course. The apparently synchronous appearance of Ag^+ and latently infected cells between 48 h and 72 h after infection suggests that the molecular pathways leading to latency and productive infection may diverge at an early stage, even when HSV gene expression has not been precluded by a lesion in the viral genome. However, on the basis of daily sampling, the possibility cannot be

Table 5.1. Synchronous appearance of productively and latently infected neurons

Day after infection	Number of neuronal profiles			
	Examined	Ag^+ (total)	LAT^+ Ag^-	LAT^+ Ag^+
2	24840	0	0	0
3	15456	175	3	25
4	13524	596	5	68
5	6486	390	22	75
45	9174	0	329	0

Figure 5.1. Analysis of the establishment of HSV latency by combined immunohistochemical detection of viral antigen and ISH for LATs. (a) Photomicrograph (x400) showing several Ag^+ neurons (brown areas) and a single Ag^- LAT^+ neuron (dense collection of grains, arrowed) in a spinal ganglion 3 days after inoculation of flank skin. (b) Photomicrograph (x1000) showing different outcomes of infection in adjacent neurons. The collection of silver grains (approx 350) over the LAT^+ neuron (arrow) represents approximately 10^3 copies of LAT. The number of grains over surrounding cells (20 to 30/cell) typifies the level of background associated with uninfected material. (c) High power (x1000) photomicrograph of an Ag^+ LAT^+ neuron, with 156 autoradiographic grains.

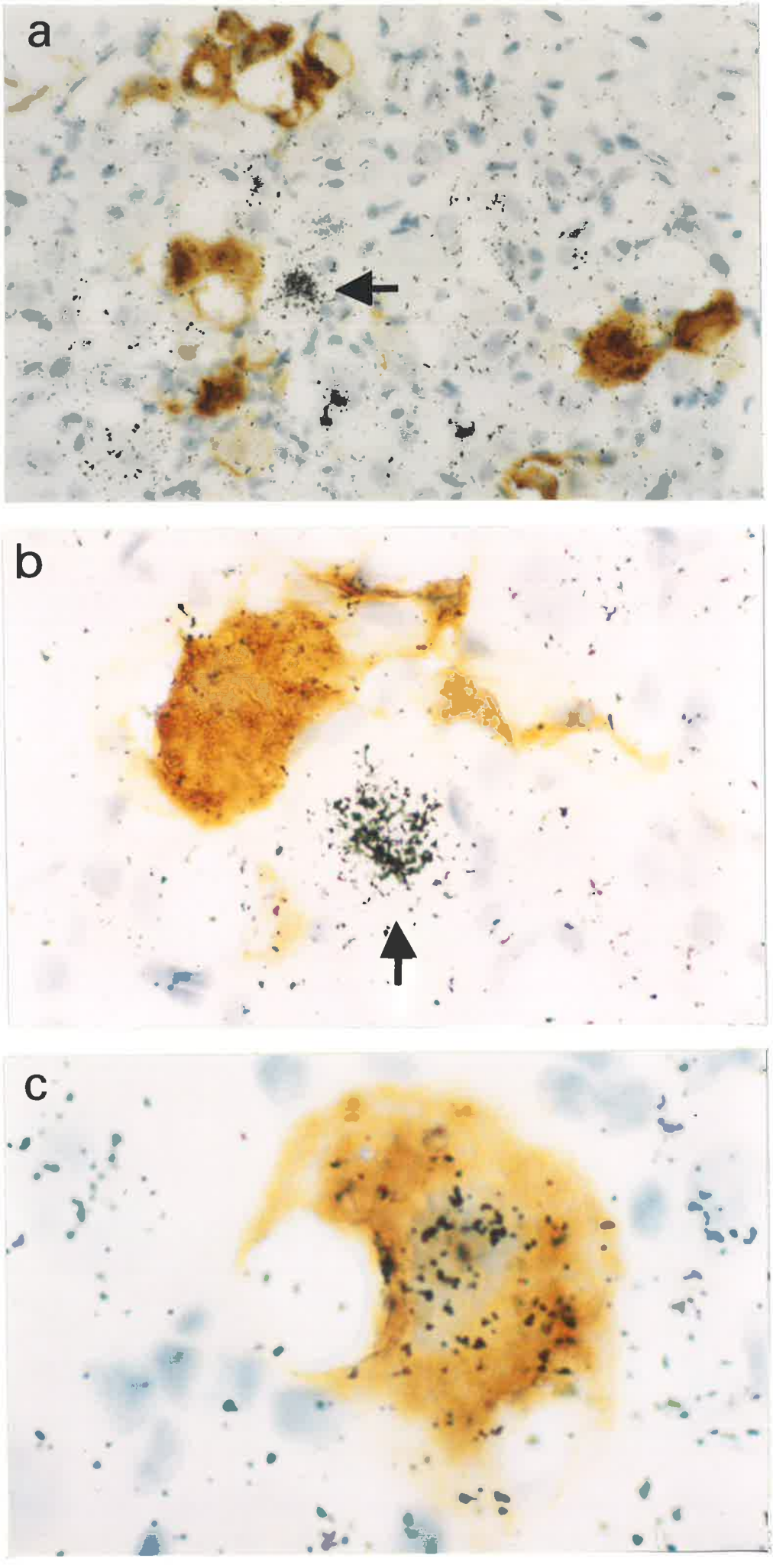
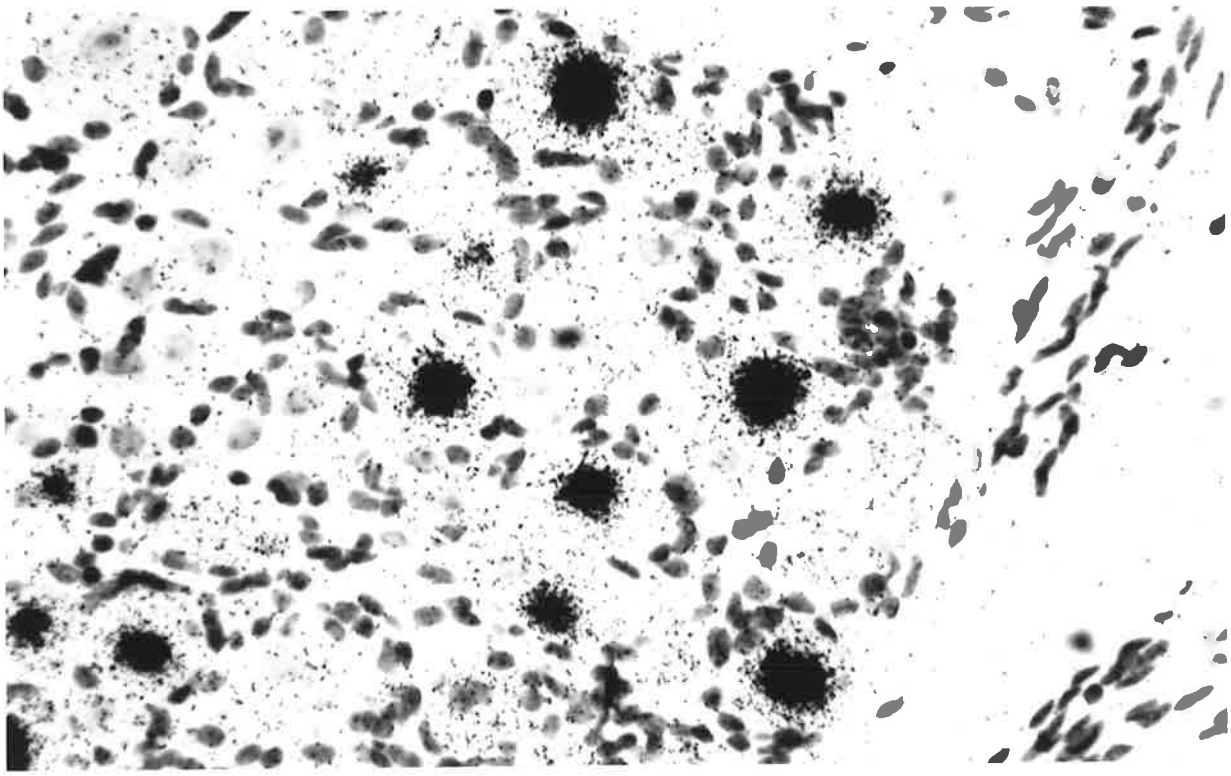

a
b
c

Figure 5.2. Analysis of ganglionic sections (x400) by ISH 45 days after infection showed that many neurons contained $>10^4$ copies of LATs (dense black areas) in contrast to the weaker signals obtained during the establishment phase (Fig 5.1b).



excluded that LAT^+ Ag^- cells were generated by a transient burst of gene expression that went undetected because it fell, in all animals tested, between days 2 and 3. The presence of Ag^+ LAT^+ neurons demonstrates that the probe used for ISH was able to penetrate immunohistochemically stained cells and is consistent with the observation that productively infected cultured cells express LATs at high multiplicity of infection (Spivack and Fraser, 1988a).

There are three possible reasons for the observed increase in the number of Ag^- LAT^+ neurons between the onset and peak of acute infection. First, this increase parallels the rising number of Ag^+ cells and may reflect the generation, by viral spread, of new sites of latency. Second, temporal accumulation of LATs (Spivack and Fraser, 1988b) may render latently infected neurons, present from the outset, more readily detectable as time progresses. Finally, although studies based on mutant and virulent strains of HSV indicate that production of viral polypeptides is not essential for establishment of latency, it does not follow that expression of viral genes necessarily precludes subsequent entry into a latent form of infection.

The presence of Ag^+ LAT^+ neurons during the establishment phase of latency is consistent with this finding and supports the possibility of an additional pathway leading to the persistence of viral genomes. In this respect the strikingly large amount of viral DNA that can be recovered from latently infected tissue merits discussion. Neither the observations reported here with

virulent HSV-1, nor those showing that replication-defective mutants, provide a straightforward explanation for the presence of up to several hundred copies of the viral genome for each latently infected neuron (Rock and Fraser, 1983; Efstathiou *et al*, 1986). Whether HSV genomes are amplified by cellular factors, as suggested (Sears and Roizman, 1990), or by viral mechanisms, or both, remains to be shown. Further dissection of viral gene activity during the establishment phase using combined immunohistochemistry and ISH, as described here, may provide an initial approach to these questions.

6. DISCUSSION

In this chapter a brief consideration will be made of the strategies employed by viruses to maintain lifelong infection and, where relevant, parallels drawn to HSV latency. For infection to persist viruses must avoid immune surveillance, and strategies used to achieve this aim are diverse. They include antigenic variation in the individual host e.g. visna virus (Narayan *et al*, 1977), induction of immunosuppressive factors e.g. Friend virus (reviewed by Dent, 1972), down regulation of viral antigen production e.g. lymphocytic choriomeningitis virus (LCMV; Joseph and Oldstone, 1975) and down regulation of host major histocompatibility complex (MHC) expression e.g. adenovirus type 12 (Bernards *et al*, 1983). In addition to evading immunity, persistence requires moderation of virus-induced cytopathology. Some viruses are able to achieve a steady state of replication without killing their host cell e.g. LCMV, while others, such as adeno-associated viruses (AAVs) and herpesviruses establish true latency in which the replicative cycle is temporarily interrupted.

DNA viruses that establish true latency merit further consideration in relation to the findings present in this thesis. Perhaps the best characterized system of virus latency is that of lambda (reviewed in Lewin, 1987). When a free bacteriophage lambda particle encounters a susceptible host cell, the linear viral DNA genome is injected into the cell where it is circularized by host enzymes. Viral genes are then expressed, like HSV, in a temporally regulated cascade, with genes

designated immediate early, early, or late. Replication of viral DNA is followed by assembly of progeny virus particles which are liberated from the cell by lysis. However in some cells there is a diversion from the lytic pathway with expression of genes that lead to integration of latent viral DNA into the cellular genome (lysogeny).

RNA transcription is initiated at two immediate early promoters, P_L and P_R. Early genes are positioned immediately downstream of immediate early genes and, by antitermination, early gene transcripts are read from P_L and P_R. Associated with each of these promoters are operators (O_L, O_R) that bind a repressor protein (the cI early gene product) responsible for prevention of lytic cycle transcription. Repressor activity therefore inhibits the lytic cycle. The dimeric structure of the repressor protein is essential for its binding to operators. An antirepressor (the Cro gene product) competitively binds to the operator regions preventing repressor action. Therefore the Cro and CI gene products are antagonistic. The critical effector over the switch between lysogency and lysis is the delayed-early protein CII, which influences the competitive binding of CI and Cro to operator regions. The state of the host cell can influence the viral lysis/lysogeny switch. For example host proteases that degrade CII are activated by growth on rich medium, so bacteriophage lambda tends to lyse cells that are growing well, but is more likely to enter lysogeny in cells that are starving and which may lack components necessary for efficient lytic growth. In a process known as induction, the bonds maintaining the dimeric structure of the repressor protein are cleaved by a

protease, releasing the repressor from the operators leading to lytic gene expression.

Lysogeny of phage lambda involves site-specific integration of viral DNA into the cellular genome. Integration requires a phage-encoded protein, Int (Nash *et al*, 1977) and a host protein (integration host factor, IHF). Integration occurs by recombination involving sites called att P, on the phage chromosome, and att B, on the cellular chromosome. Some comparisons can be drawn between HSV latency and lambda lysogeny. In each case viral genomes circularize on entry into cells. However results presented in this thesis show HSV can enter latency without detectable gene expression, whereas the lysis/lysogeny decision of phage lambda is taken after expression of immediate early genes. Further, lambda DNA integrates into the cellular genome whilst there is no evidence of integration of HSV genomes into cellular DNA. Control of phage gene expression has been characterized much more extensively than in HSV. In particular the mechanism of phage induction is known whereas how HSV reactivates remains obscure.

The discovery that adeno-associated viruses (AAV), defective parvoviruses, persist in cells of primate origin and can be reactivated by superinfection with helper viruses, led to the establishment of latently infected cell lines which are amenable to molecular analysis (Hoggan *et al*, 1972). These viruses are unable to enter lytic infection in the absence of a helper virus, e.g. an adenovirus or a herpesvirus (Atchison *et al*, 1965; McPherson and Rosenthal, 1985) although recently it has

been shown that in cells pretreated with certain toxic agents AAVs can replicate without help (Schlehofer *et al*, 1986; Yakobson *et al*, 1987). In the absence of helper virus coinfection, the AAV virion can penetrate the nucleus where its ~4.7 kb single-stranded DNA genome is uncoated and latency is established, like HSV, in the absence of detectable virus gene expression (reviewed in Berns, 1990). Southern hybridization analysis of AAV DNA in latently infected human cell lines showed that it integrates into cellular DNA with the 145 nucleotide inverted terminal repeats of the virion positioned at the junctions with cell DNA (Handa *et al*, 1977; Cheung *et al*, 1980). Following high multiplicity infection of a human cell line with AAV, latent infection was established that could be reactivated by adenovirus superinfection for more than 100 passages of the cell line. In the absence of adenovirus, cells were free of AAV antigen and infectivity. In one report the AAV genome was shown to preferentially integrate at a specific site in the q arm of chromosome 19 (Hotin *et al*, unpublished data, cited in Berns, 1990). This is the first evidence for site-specific integration of any eukaryotic virus.

Possibly the best characterized persistent infection by a herpesvirus is that caused by Epstein Barr virus (EBV) which is carried by more than 95% of adults as a largely asymptomatic infection (reviewed in Miller, 1990). Primary infection with EBV typically occurs through salivary exchange resulting in virus replication in oropharyngeal epithelial cells which it is thought leads to virus persistence in a non-replicating form in B lymphocytes (Stevens, 1989). Evidence that B cells

harbour the latent virus comes from the observation that cultures of circulating lymphocytes, from previously infected individuals, show spontaneous transformation to EBV genome-positive lymphoblastoid cell lines (Rocchi *et al*, 1977). *In vitro* the virus can transform, or immortalize, B cells which become either productively or non-productively infected. Non-producer cell lines harbour the virus in a latent form from which reactivation can be induced following a variety of external stimuli. Such *in vitro* systems have been used as models for EBV latency and have enabled molecular characterization of latent viral genomes. Latent EBV genomes are characteristically present as covalently closed circular molecules in multiple copies per latently infected B cell (zur Hausen *et al*, 1970; Nonoyama and Pagano, 1971; Kawai *et al*, 1973; Lindahl *et al*, 1976). EBV resembles HSV in that replication of the viral genome is not a requirement either for establishment of the latent state or for transformation of cells and in that these phenomena require circularization of incoming genomes (Hurley and Thorley-Lawson, 1988). In culture the number of viral genomes per cell remains constant over time, implying a tightly controlled replicative system that is co-ordinated with cell division (Stevens, 1989). There is evidence that in certain cell lines e.g. Nomalawa, EBV DNA may be integrated into the cellular genome (Henderson *et al*, 1983; Matsuo *et al*, 1984). Transformed cells harbouring EBV genomes express one or more of a group of virally encoded proteins, the genes for which are dispersed throughout the genome, including six nuclear antigens (designated EBNA -1, -2, -3a, -3b, -3c, and -LP) and three membrane proteins (latent membrane proteins, LMP - 1, -2A, and -2B) (reviewed in Kieff and Liebowitz, 1990).

Studies on EBV-positive cell lines have identified three distinct forms of virus latency (Rowe *et al*, 1992) exemplified by different patterns of gene expression detected during experimental induction of the lytic cycle. In the first form of latency, only EBNA1 is expressed; in the second, all nine viral proteins are expressed; and in the third, EBNA-1 and LMPs are expressed in the absence of other EBNAs.

The functions of the EBV gene products expressed in cells harbouring the virus remain largely unknown. EBNA-1 and a *cis*-acting sequence, *oriP*, (a putative origin of plasmid replication) together constitute a plasmid replicon which ensures replication and maintenance of the viral genome in a proliferating cell population (Baichwal and Sugden, 1988). EBNA-1 also trans-activates at least one latency-specific EBV promoter (Sugden and Warren, 1989). EBNA-2 transcriptionally transactivates LMP-1 as well as cellular genes that are believed to play a role in EBV-induced B cell growth transformation such as the proto-oncogene *c-fgr* and the gene encoding the B-cell activation antigen CD23 (Wang *et al*, 1987; Abbot *et al*, 1990). All other EBNAs are thought to be transcriptional trans-activators except EBNA-LP (Kieff and Leibowitz, 1990). LMP-1 is required for activation of human B-lymphocytes (Wang *et al*, 1990), can protect lymphoblastoid cell lines from apoptosis (Henderson *et al*, 1991) and is defined as a viral oncogene because of its ability to transform cultured rodent fibroblasts (Wang *et al*, 1985). LMP-2A and -2B associate with LMP-1 in the immortalization of lymphoid cells by interacting with a cellular tyrosine kinase (Longnecker *et al*, 1991). It has

been suggested that whilst EBNA-1 is essential for maintenance of the EBV genome in proliferating cells, other EBNAs and the LMPs are required for growth transformation and immortalization of infected B cells (Baichwal and Sugden, 1988; Garcia-Blanco and Cullen, 1991). The observation that EBNA-2 and LMP-1 can provide targets for EBV-induced cytotoxic T-lymphocytes (Murray *et al*, 1988) has led to the proposal that latency specific EBV gene expression *in vivo* might be more restricted than gene expression found *in vitro* (Klein, 1989).

Whilst the molecular nature of activation of EBV replication *in vivo* is obscure, EBV replication can be activated *in vitro* by a number of substances including antibody to immunoglobulin and 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (Liebowitz and Kieff, 1990), which are thought to act by inducing expression of the EBV immediate-early gene BZLF-1 (Miller, 1990). The product of BZLF-1 up regulates its own expression in a positive feedback loop and induces expression of other viral immediate early genes, thereby initiating the lytic cascade of EBV gene expression. Spontaneous reactivation *in vitro* occurs only 1 in 10^3 to 10^6 B cells, suggesting that activation of the BZLF-1 gene occurs rarely. The recognition that a single viral gene product may control the EBV latency-to-replication switch offers promise of a detailed understanding of a central process in virus persistence.

Latency associated transcription from the genomes of the alphaherpesviruses bovine herpesvirus 1 (BHV-1) (Fig 6.1) and pseudorabies virus (PRV) (Fig 6.2)

Figure 6.1. Schematic representation of the genome of bovine herpesvirus 1 (BHV-1). The genome is organized into unique long (U_L), internal repeat (I_R), unique short (U_S) and terminal repeat (T_R) regions. The approximate size and location of a transcript produced during latency (latency related RNA: LR RNA) is shown. A 2.9 kb RNA molecule is transcribed during lytic infection from the complementary strand with immediate-early kinetics.

BOVINE HERPESVIRUS 1

GENOME STRUCTURE

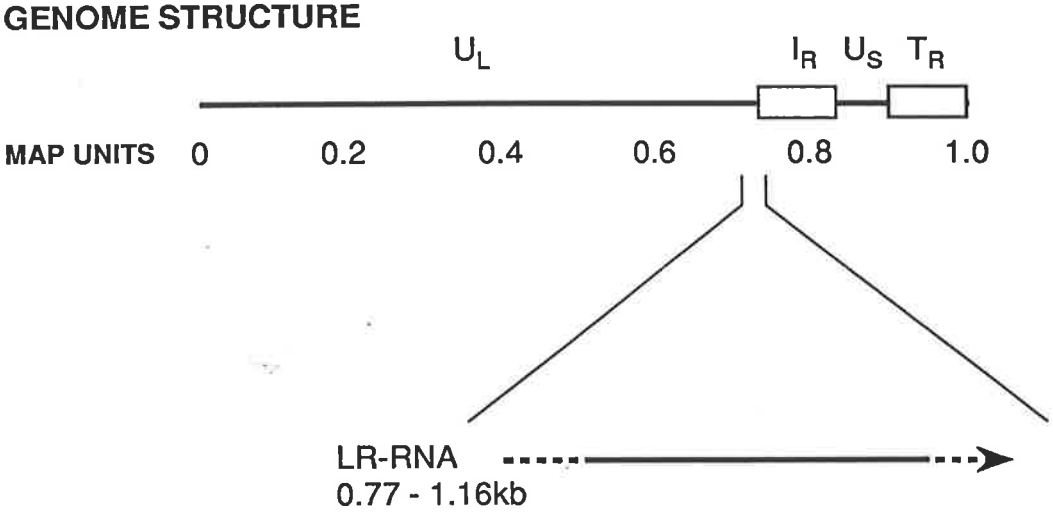
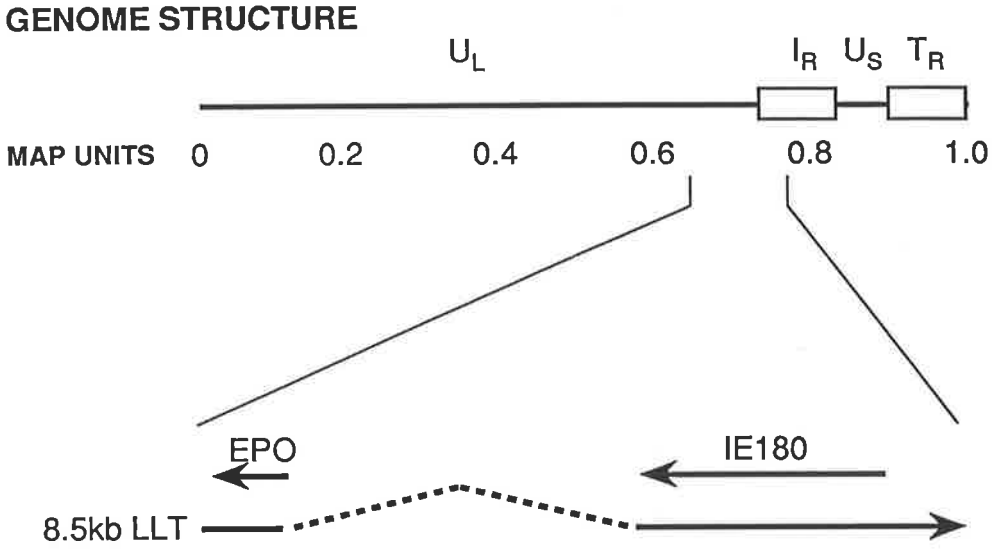


Figure 6.2. Schematic representation of the genome of pseudorabies virus (PRV). The genome is organized into the unique long (U_L), internal repeat (I_R), unique short (U_S) and terminal repeat (T_R) regions. 8.5 kb of the genome, designated the long latency transcript (LLT), is transcriptionally active during latency, is spliced to yield smaller transcripts, and is complementary to the only known PRV immediate-early gene, IE180, and to early gene EP0.

PSEUDORABIES VIRUS

GENOME STRUCTURE



is markedly similar to that of HSV-1. In the case of BHV-1, a 0.77 to 1.16 kb transcript designated the latency related RNA (LR RNA) is present in trigeminal ganglia of experimentally infected rabbits, and a similar transcript has been detected in trigeminal ganglia of cattle. The transcript, which contains predicted open reading frames, is opposite in sense to, and maps to the same area, as a region of the viral genome that is expressed with immediate early kinetics during lytic infection (Kutish *et al*, 1990). The functional significance of the latency-related transcription of BHV-1 remains unknown. With respect to PRV ~8.5 kb of the genome, designated the long latency transcript (LLT), is transcriptionally active during latency (Cheung, 1991) and within this region small transcripts of 2.0 and 0.95 kb have been detected (Cheung, 1989). These transcripts overlap and are opposite in polarity to a region that is expressed in lytic infection with immediate early kinetics. Polyadenylated and non-polyadenylated PRV LATs have been described (Cheung, 1990, 1991; Priola *et al*, 1990). The function of these transcripts remains unknown.

Latent infection with varicella zoster virus (VZV) has not been extensively characterized, perhaps because the lack of an animal model restricts study to tissue samples taken from cadavers. Several lines of evidence suggest that sensory ganglia harbour the latent virus: (i) nucleic acid sequences have been detected in PNS tissue by *in situ* and Southern hybridization (Croen *et al*, 1988; Gilden *et al*, 1983, 1986; Hyman *et al*, 1983). (ii) VZV gene products have been detected in ganglia following explant culture (Vafai *et al*, 1988). (iii) zoster

lesions have a characteristic dermatomal distribution. Controversy exists regarding the cell type harbouring the latent virus: on the basis of *in situ* hybridization, neurons have been proposed as the site of latency (Hymen *et al*, 1983; Gilden *et al*, 1986) as have the capsular cells (Croen *et al*, 1988). At least five regions of the viral genome have shown evidence of transcription during latency (Croen *et al*, 1988). As with other herpesviruses, the purpose of transcription during latency is not known.

It is clear that further understanding of HSV latency is dependent on elucidation of the molecular switches that determine whether HSV, on entry to a neuron, lyses the cell or establishes latent infection. Evidence presented in this thesis suggests that this switch can operate during the earliest stages of the replicative cycle. Detailed understanding, at the molecular level, of events underlying establishment and maintenance of HSV latency may have to await the development of an *in vitro* model which effectively mimics neuronal latency. In the meantime, animal studies continue to provide the means to study pathogenesis of HSV.

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