ANALYSIS OF THE LATENCY ASSOCIATED TRANSCRIPTS OF HERPES SIMPLEX VIRUS TYPE 1

JANE LOUISE ARTHUR
BSc (Hons)

Division of Medical Virology
Institute of Medical and Veterinary Science
Adelaide, South Australia

Department of Microbiology and Immunology
University of Adelaide
Adelaide, South Australia

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CONTENTS

ABSTRACT

This thesis reports a novel approach to the study of HSV-1 transcripts during latency, based on accumulating evidence that macromolecular complexes involved in the synthesis and processing of cellular and viral RNAs occupy discrete domains in cell nuclei. A method for high resolution non-isotopic *in situ* hybridization (ISH) was developed to study the intracellular location of HSV-1 latency associated transcripts (LATs) in primary sensory neurons of latently infected mice and humans. Minor LATs were localized to sharply defined intranuclear foci of 1-3 μm diameter. On average, there were 2.6 to 2.8 foci per LAT^+ neuronal profile (5 μm), representing about 10 to 11 foci per cell. In contrast, major LAT sequences were diffusely distributed throughout the nucleoplasm of latently infected neurons, with prominent sparing of nucleolar regions. Similar staining patterns were observed in latently infected human trigeminal ganglia. Thus, high and low abundance transcripts could be readily differentiated according to their spatial distribution.

Using a mixture of major LAT- and minor LAT-specific probes the characteristic staining patterns could be superimposed, demonstrating for the first time that these RNAs co-exist in latently infected neurons. In these experiments foci were readily distinguished from a background of diffuse staining however, focal staining was not a feature of probes complementary to sequences wholly within the major LAT region. The molecular basis for the segregation of major and minor LATs shown in these experiments is not clear. The data is consistent with generation of major LATs through splicing of a large primary LAT species but does not exclude the possibility that the 2 kb major LAT is a primary transcript initiated 0.7 kb downstream of the LAT promoter LAP1.

The ability to delineate the subcellular location of major and minor LATs by ISH establishes a foundation for studying the generation and transport of these molecules *in vivo*. To initiate these studies, a method for detection of nuclear antigens in sections of frozen murine sensory ganglia was developed and the distribution of cellular antigens associated with mRNA splicing in the nuclei of neurons *in vivo* was characterized.

DECLARATION OF ORIGINALITY

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

I give my consent to this copy of my thesis, when deposited in the university library, being made available for loan and photocopying

JANE LOUISE ARTHUR

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ABBREVIATIONS

ANA	anti-nuclear antibody
α-TIC	alpha transinducing complex
α-TIF	alpha transinducing factor
anti-DIG-AP	alkaline phosphatase conjugated anti-digoxigenin Fab fragments
anti-DIG-POD	peroxidase conjugated anti-digoxigenin Fab fragments
anti-DIG-FITC	fluorocein conjugated anti-digoxigenin Fab fragments
BHV-1	bovine herpes virus type 1
Cat#	catalogue number
cAMP	cyclic adenosine monophosphate
CMV	cytomegalovirus
cDNA	complementary DNA
CNS	central nervous system
CRE	cAMP responsive element
CREB	cAMP responsive element binding site
Da	Dalton
DDW	double distilled water
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dl	deletion
drg	dorsal root ganglia
DIG	digoxigenin
EAA	ethanol acetic acid
EBV	Epstein-Barr virus
EHV-1	equine herpes virus
Fab	fragment (ab) region of immunoglobulin
FCS	foetal calf serum
FISH	fluorescence *in situ* hybridization
FITC	fluoroscein-isothyocyanate
G	gravity
g	glycoprotein
HeLa	human epitheloid cervical carcinoma cells
HHV-6	human-herpesvirus 6
HHV-7	human-herpesvirus 7
hnRNP	hereonuclear ribonucleoprotein
hr	hour
hsr	heat shock response
hsps	heat shock response proteins
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpes virus saimiri
ICP	infected cell polypeptide
IE	immediate early
ISH	*in situ* hybridization
ip	intraperitoneal
IR_L	internal repeat flanking U_L
IR_S	internal repeat flanking U_S
kb	kilobase
kDa	kiloDalton
LAA	latency associated antigen
LAP1	latency associated promoter element 1

LAP2	latency associated promoter element 2
LAT	latency associated transcript
LAT^+	LAT positive
LAT^-	LAT negative
LLT	large latency transcript
LPBF	latency promoter binding factor
LR	latency related
MDV	Marek's disease virus
MEL	murine erythroleukemia
min	minute
mM	millimolar
mRNA	messenger RNA
μg	micrograms
μm	micrometre
μl	microlitre
nm	nanometer
ori	origin of replication
ORF	open reading frame
p	plasmid
pac	packaging signal
4%PB	4% paraformaldehyde buffer
PCR	polymerase chain reaction
pfu	plaque forming units
PLP	periodate-lysine paraformaldehyde
$poly(A)^+$	polyadenylated
pre-mRNA	precursor mRNA
PRV	pseudorabiesvirus
rATP	riboxy-adenosine-5'-triphosphate
rCTP	riboxy-cytosine-5'-triphosphate
rGTP	riboxy-guanosine-5'-triphosphate
rUTP	riboxy-uridine-5'-triphosphate
RNA	ribonucleic acid
RNAse	ribonuclease
rRNA	ribosomal RNA
sec	second
sm	antigen associated with splicing snRNPs
snRNP	small nuclear ribonucleoprotein
T_R	terminal repeat
U_L	long unique region
U_S	short unique region
vhs	virion host shutoff
VP	virion polypeptide
Vmw	virion protein molecular weight
Xic	X chromosome inactivation centre

1 INTRODUCTION AND LITERATURE REVIEW

1.1 HERPESVIRIDAE

Herpesviruses are double stranded DNA viruses of eukaryotes capable of establishing lifelong latent infection in the host organism (for review see Honess, 1984 and Roizman, 1990). There are over 100 known members of the herpesviridae of which seven, herpes simplex viruses 1 and 2 (HSV-1, HSV-2); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); cytomegalovirus (CMV), human herpes virus 6 (HHV-6) and human herpes virus 7 (HHV-7), naturally infect humans.

1.1.1 Biological properties

Herpesviruses share a number of structural and biochemical features. All have a common virion architecture consisting of (a) an electron opaque core containing a single linear genome of ≥120 kb (b) an icosadeltahedral capsid, approximately 100 nm in diameter, consisting of 162 prismatic capsomeres (c) an amorphous tegument of viral proteins surrounding the capsid and (d) an outer lipid envelope with external glycoprotein spikes (Wildy *et al*, 1960; Honess and Watson, 1977; Roizman *et al*, 1981).

At the molecular level herpesviruses share some rudimentary mechanisms of gene regulation and all specify a large number of the enzymes necessary for nucleic acid metabolism and DNA synthesis. Further, synthesis of viral DNA and assembly of capsids occurs in the nucleus with the production of infectious progeny virus, at least in cell culture, invariably accompanied by irreversible destruction of the host cell. The central feature of herpesviruses is their capacity to establish latent infection of their natural hosts in which only a small subset of viral genes are expressed (for review see Roizman, 1990).

There are three subfamilies (α, β, γ) within the herpesviridae, distinguished on the basis of biological properties such as host range and tissues involved in acute and latent infections *in vivo*; by host range, growth rate and cytopathology of infections in cell culture and G+C nucleotide content (Barahona *et al*, 1974; Honess and Watson, 1977; Roizman *et al*, 1981). Comparison of genome organization and amino acid sequence homology for individual genes has, in general, supported classification based on biological properties although there are some exceptions eg., Marek's disease virus (MDV) which is biologically a γ-herpesvirus yet its genetic structure and sequence is more similar to α-herpesviruses (Buckmaster *et al*, 1988).

1.1.2 Alphaherpesvirinae

Members of this subfamily characteristically display a broad host range, relatively short and generally cytocidal reproductive cycles ($\geq$18 hr), rapid spread of infection in cell culture and the ability to persist in a latent form frequently, but not exclusively, in sensory ganglia (Roizman *et al*, 1981). Primary infection *in vivo*, occurs in cells of ectodermal origin in the skin, eyes, oral cavity, genital and respiratory tracts, and may give rise to vesicular lesions. In their natural hosts alphaherpesviruses are capable of productive infection of a wide range of tissue and cell types. Fulminant disseminated infections may occur in neonates and immunocompromised adults of natural host species with potentially fatal consequences. During primary infection virus ascends sensory nerves innervating the inoculation site and establishes a latent infection within ganglia, from which virus may reactivate periodically causing recurrence of disease (Roizman *et al*, 1981; Honess, 1984).

Alphaherpesvirinae may be further scissioned into agents with greater resemblance to HSV (*Simplexviruses* or α_1 eg., infectious bovine herpes virus 1, BHV-1 and equine herpes virus

1, EHV-1) and agents with greater similarity to VZV (*Varicelloviruses* or α_2 eg., pseudorabies virus, PRV) (Honess, 1984 and Roizman, 1990).

1.1.3 Betaherpesvirinae

Known also as cytomegaloviruses, members of this subfamily are characterized by narrow host range, relatively protracted reproductive cycle (≥72 hr) and slowly progressing lytic foci *in vitro* with infected cells often displaying enlargement (cytomegaly) (Wright, 1973). Cytomegalovirus infected cells produce and release several forms of noninfectious enveloped particles composed of virus tegument and envelope polypeptides (eg., dense bodies, Craighead *et al*, 1972; Irmiere and Gibson, 1983).

The biologically important site of cytomegalovirus persistence is not clear although latent infections may be established in secretory glands, lymphoreticular cells and kidneys (Roizman *et al*, 1981). Persistent infection with these viruses is usually asymptomatic, but they may produce disseminated infection and severe disease in neonates or immunocompromised adults of their natural host species (Honess, 1984).

Betaherpesvirinae may be further divided into two genera, (a) *Cytomegaloviruses*, including human CMV and (b) *Muromegaloviruses*, containing murine CMV (murid herpes virus 1, MHV-1) (Melnick, 1990).

1.1.4 Gammaherpesvirinae

The host range of gammaherpesviruses is generally limited to the same family or order as the natural host (Roizman *et al*, 1981). All members of this subfamily replicate in

lymphoblastoid cells *in vitro* and some also cause lytic infections in a number of epithelioid and fibroblastoid cells (Roizman *et al*, 1981). Characteristically these viruses cause a mild, transient lymphoproliferative disease in their natural hosts and have the capacity to infect and persist as replicating episomes, in the absence of general virus gene expression, in dividing populations of, eg., lymphoid cells (Roizman *et al*, 1981; Honess, 1984). A member of this group, Epstein-Barr virus (EBV), is a causative agent of infectious mononucleosis in humans (Henle *et al*, 1968). Gammaherpesviruses may also cause neoplasia eg., EBV immortalizes lymphocytes *in vitro* (Pattengale *et al*, 1973) and is associated with malignancies *in vivo,* such as Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma (Miller, 1990).

Gammaherpesviruses are divided into two genera on the basis of cell tropism for latent infections and genetic complexity, (a) B-lymphotrophic *Lymphocryptoviruses* (or γ_1-herpesviruses) which include EBV and (b) T-lymphotrophic *Rhadinoviruses* (or γ_2-herpesviruses) which include HVS (Honess, 1984; Melnick, 1990).

1.2 HERPES SIMPLEX VIRUSES

Herpes simplex viruses consist of two closely related, but serologically distinguishable agents (Schneweiss, 1962), designated type 1 (HSV-1) and type 2 (HSV-2). HSV-1 and HSV-2 display similar pathology and colinear genome organization with ~50% homology at the nucleotide level (Kieff *et al*, 1972). These viruses cause a wide spectrum of clinical disease including one of the most common of all human viral diseases, "cold sores" or "fever blisters" which may recur at frequent intervals throughout the life of the host.

1.2.1 Components of the HSV-1 virion

Genome

The genome of the mature virion is a linear molecule of ~152 kb consisting of two covalently joined fragments of ~126 and ~26 kb, designated the long (L), and short (S) components respectively (McGeoch *et al*, 1985, 1986, 1988; Perry and McGeoch, 1988). L and S fragments each contain a unique sequence (U_L and U_S respectively) flanked by inverted repeats, designated ab and b'a' flanking U_L and c'a' and ca flanking U_S (Sheldrick and Berthelot, 1974; Wadsworth *et al*, 1975). The prototype genome (designated P) may thus be represented as: ab-U_L-b'a'c'-U_S-ca (Wadsworth *et al*, 1975). During viral replication the L and S components freely invert generating four isoforms in equimolar amounts (Hayward *et al*, 1975; Delius and Clements, 1976; Clements *et al*, 1976). However, neither the internal repeat sequences nor the capacity to invert are essential for growth of virus in cell culture (Poffenberger *et al*, 1983; Jenkins and Roizman, 1986).

The complete sequence of HSV-1 (strain 17) has been determined and open reading frame analysis indicates that there are at least 73 potential protein-coding genes of which three, ICP0, γ34.5 and ICP4 are diploid, being located in the inverted repeats (McGeoch *et al*, 1985, 1986, 1988; Chou and Roizman, 1986; Perry and McGeoch, 1988). The functions of ~50 genes have been determined of which, individually, thirty are dispensable for productive infection in standard cell lines (reviewed in McGeoch *et al*, 1993), although many of these mutant viruses grow less efficiently than wildtype. Some "dispensable" proteins are however, required for pathogenic infection in animal models (eg., thymidine kinase, Field and Wildy, 1978; Efstathiou *et al*, 1989 and γ34.5, Chou *et al*, 1990).

Capsid

On electron microscopic examination, capsids display an external icosahedral shell surrounding an electron opaque core of uniform, densely stained areas of DNA which entirely occupy the capsid interior (Schrag *et al*, 1989; Booy *et al*, 1991). The popular hypothesis that the DNA of the core is arranged as a toroid around a central protein 'plug' (Furlong *et al*, 1972) has been challenged (Puvion-Dutilleul *et al*, 1987). Rather, virion DNA may exist in a liquid crystalline state, similar to that suggested for DNA bacteriophages λ and T7 (Booy *et al*, 1991; Lepault *et al*, 1987).

Tegument

The capsid is embedded in an amorphous mass of viral proteins, designated 'tegument', which shows variable size and shape on electron microscopic examination (Roizman and Furlong, 1974). Little is known about the structure or function of the tegument although, as transfected virion DNA is capable of initiating productive infection, it can be concluded that tegument proteins are not an absolute requirement for viral replication if large numbers of genomes are artificially introduced into cells (Graham *et al*, 1973; Sheldrick *et al*, 1973). The functions of several proteins found in the tegument have, at least in part, been defined including the virion host shut-off (*vhs*) a protein which is encoded by U_L41 (Kwong *et al*, 1988) and is responsible for destabilization and degradation of host mRNA (Becker *et al*, 1993) and thus shutdown of host protein synthesis. A second tegument protein Vmw65 (also termed virion protein 16 (VP16) or α transinducing factor (α-TIF)), present in ~1000 copies per virion (Heine *et al*, 1974), forms, in association with cellular factors, a potent transcription complex (α trans-inducing complex, α-TIC) capable of inducing expression of a subset of viral genes (see section 1.2.3: *viral gene expression*; Post *et al*, 1981; Batterson and Roizman, 1983; Campbell *et al*, 1984; Kristie and Roizman, 1987; O'Hare *et al*, 1988). Further viral transcription activators, infected cell

polypeptides (ICPs) 4 and 0, have been detected in tegument (Yao and Courtney, 1989, 1992; McLauchlan and Rixon, 1992) although the significance of such observations has not been determined. The presence of transcription regulatory factors in the tegument indicates that this virion component may play an important role in the initiation of infection.

Envelope

The virion envelope consists of virus-encoded glycoprotein spikes embedded in a lipid membrane of cellular origin (Wildy *et al*, 1960; Asher *et al*, 1969; Stannard *et al*, 1987). To date ten membrane glycoproteins have been identified (designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL), seven of which have been identified as components of the virion envelope (all but gJ, gK, gL) (Spear, 1993). None of the glycoproteins has been shown to be necessary for virion assembly or envelopment (Rixon, 1993) although several are required for virion adsorption and uptake (for review see Spear, 1993 and section 1.2.2)

1.2.2 Infection and replication in cell culture

Productive HSV-1 infection *in vitro* may be considered in five stages, namely (a) attachment and entry, (b) translocation of viral DNA to the nucleus, (c) viral gene expression, (d) replication of viral DNA, (e) virion assembly and egress.

Attachment and entry

The events and molecules involved in virus attachment and penetration are not yet fully elucidated. HSV appears to have multiple mechanisms, of varying efficiencies, for binding to cells and from the information currently available about the molecular interactions required for alphaherpesvirus entry into cells, the process appears to be similar for HSV-1, HSV-2, PRV and BHV-1 (Spear, 1993). The initial interaction between the virus and cell

that promotes efficient virus entry is binding of viral membrane glycoprotein C (gC) to cell surface proteoglycan heparin sulphate moieties (WuDunn and Spear, 1989; Campadelli-Fiume *et al*, 1990; Herold *et al*, 1991; Sheih *et al*, 1992; Gruenheid *et al*, 1993). Following adsorption, the virion envelope may fuse with the plasma membrane (Morgan *et al*, 1968; Para *et al*, 1980; Fuller and Spear, 1987; Fuller *et al*, 1989; Johnson *et al*, 1984) releasing the nucleocapsid directly into the cytoplasm. Although uptake of HSV-1 by endocytosis (a general mechanism by which enveloped viruses may enter cells; Marsh and Helenius, 1989) has been observed (Holmes and Watson, 1961; Dales and Silverberg, 1969), various agents which block endocytosis have no effect on the infectious entry of HSV-1 into cells (Koyama and Uchida, 1984; Wittels and Spear, 1991). Further, endocytosis may largely be a dead-end route leading to virus degradation in lysosomes (Campadelli-Fiume *et al*, 1988). Although the pathway of virus entry has not been determined, at least three viral glycoproteins gB, gD and gH are essential for the penetration into cells (Sarmiento *et al*, 1979; Little *et al*, 1981; Cai *et al*, 1988; Fuller and Spear, 1987; Highlander *et al*, 1987; Campadelli-Fiume *et al*, 1988; Ligas and Johnson, 1988; Desai *et al*, 1988; Fuller *et al*, 1989, Forrester *et al*, 1992). One or more of these glycoproteins may interact with a second, as yet undefined, cell surface receptor, facilitating fusion of the virion envelope with the plasma cell membrane.

Translocation of viral DNA to the nucleus

Fusion of the viral envelope and plasma cell membrane releases the viral nucleocapsid into the cytoplasm from where capsids are transported to nuclear pores (Tognon *et al*, 1981). This translocation is probably mediated by the cellular cytoskeleton (Kristensson *et al*, 1986). Proteins contained within the tegument may also enter the nucleus one of which, Vmw65 (Campbell *et al*, 1984), participates in transactivation of viral gene expression (reviewed by O'Hare, 1993) and a second, *vhs*, is involved in the shut-off of host

macromolecular synthesis (Strom and Frenkel, 1987; Fenwick and Walker, 1978; Kwong *et al*, 1988). Upon entry to the nucleus, the linear virion DNA is circularized either by a host enzyme or by a viral protein brought into the cell during infection (Poffenberger and Roizman, 1985).

Viral gene expression

HSV subverts host RNA biogenesis and utilizes endogenous RNA polymerase II for viral gene transcription (Costanzo *et al*, 1977). As a general rule most HSV-1 genes are transcribed from independent promoters, with post-transcriptional modifications similar to eukaryotic mRNAs, including capping, methylation and polyadenylation (Bachenheimer and Roizman, 1972). Notably, few HSV mRNAs are spliced.

Expression of HSV-1 proteins occurs in a sequential cascade of three co-ordinately regulated groups of genes (Honess and Roizman, 1973, 1974). Genes within a group have similar requirements for, as well as kinetics of, expression. These expression groups are designated α, β, and γ or, immediate-early, early, and late respectively.

Alpha genes: The first infected cell polypeptides (ICPs) expressed are the five alpha (α) proteins 0, 4, 22, 27 and 47. Peak synthesis occurs 2-4 hr after infection with some α protein production continuing throughout infection (Honess and Roizman, 1974). Expression of α polypeptides does not rely on the prior *de novo* synthesis of viral proteins (Honess and Roizman, 1974; Post *et al*, 1981) although all are upregulated by α-TIC, the transinducing complex comprised of the viral tegument protein Vmw65 and cellular proteins, which binds to a common sequence element TAATGARAT (where R is a purine residue), upstream of α genes (Campbell *et al*, 1984; Gaffney *et al*, 1985; Kristie and Roizman, 1987; O'Hare *et al*, 1988; reviewed by O'Hare, 1993). With the exception of

ICP47, α gene products have a role in regulation of all classes of HSV genes. However, only the ICPs 4 and 27 are found to be essential for replication *in vitro* (Watson and Clements, 1978; Preston, 1979 a&b; Sacks *et al*, 1985).

ICP4 is required for induction of β and γ gene expression and for repression of its own synthesis (Watson and Clements, 1978, 1980; Preston, 1979 a&b; Dixon and Schaffer, 1980; De Luca and Schaffer, 1985; Gelman and Silverstein, 1987 a&b; O'Hare and Hayward 1985 a&b, 1987; Roberts *et al*, 1988).

ICP27 upregulates γ gene expression (Sacks *et al*, 1985; Smith *et al*, 1992) and, in combination with other alpha proteins, can act as either a *trans*-activator or *trans*-repressor of gene expression (Sekulovich *et al*, 1988). ICP27 reduces the expression of spliced transcripts (Sandri-Goldin and Mendoza, 1992), possibly through a post-transcriptional mechanism involving an interaction with host splicing factors (Phelan *et al*, 1993).

ICP0 can transactivate expression from all three major kinetic classes of HSV promoters (Everett, 1984; O'Hare and Hayward, 1985 a&b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1987 a&b; Mavromara-Nazos *et al*, 1986) and although not essential for replication in cell culture (Stow and Stow, 1986; Sacks and Schaffer, 1987), it may have greater influence at low multiplicities of infection (Cai and Schaffer, 1989; Everett, 1989). Further, ICPO may have a critical role in the reactivation of HSV from latency (Leib *et al*, 1989a).

The action of ICP22 is not understood although ICP22 null mutants show restricted host range because of impaired γ gene expression in some non-primate cell lines (Sears *et al*, 1985). Little is known about the role of ICP47 except that it does not appear to be

essential for growth in cell culture (Mavromara-Nazos *et al*, 1986).

Beta genes: The second kinetic class of viral proteins are the β polypeptides, synthesized at peak rate 5-7 hr after infection, which require the presence of functional α proteins for their expression (Honess and Roizman, 1974, 1975). Many of the β proteins are involved in nucleotide metabolism and DNA replication, and viral DNA replication initiates soon after the onset of β protein synthesis (Honess and Roizman, 1974). β proteins include the replication origin binding protein (UL9), DNA polymerase (UL30) and its associated catalytic factor (UL42), single-stranded DNA binding protein (UL29/ICP8) and proteins of the helicase/primase complex (UL5, UL8, UL52).

β proteins are comprised of two expression groups (1) β_1: β proteins expressed relatively early which require, at least, the presence of ICP4 for synthesis and, (2) β_2: β proteins whose expression is delayed, with peak synthesis 6-7 hr after infection (Roizman and Sears, 1990).

Gamma genes: The expression of γ genes is detectable ~9 hr after infection. γ genes encode mainly structural proteins required for the assembly and maturation of progeny virions (Honess and Roizman, 1974; Roizman and Sears, 1990). They are further subdivided into, (1) γ_1: genes which are expressed relatively early in infection and are only marginally affected by the inhibition of DNA replication (eg., UL48 which encodes Vmw65) and, (2) γ_2: genes which are transcribed late in infection and which stringently require viral DNA replication for expression (eg., UL44 which encodes glycoprotein C) (Conley *et al*, 1981; Silver and Roizman, 1985; Mavromara-Nazos and Roizman, 1987; Roizman and Sears, 1990).

Replication of viral DNA

The viral proteins involved in DNA synthesis are mainly β gene products. DNA replication is initiated soon after the synthesis of β proteins begins (~3 hr after infection) and proceeds for at least 9 hr (Roizman *et al*, 1965; Roizman and Roane, 1964). HSV specifies a large number of proteins involved in nucleic acid metabolism and DNA replication, seven of which, UL5, UL8, UL9, UL29, UL30, UL42 and UL52, form the minimum requirements for origin-dependent initiation of replication and subsequent synthesis of DNA (Challberg, 1986; Wu *et al*, 1988, and reviewed by Challberg, 1991). Other proteins are involved in processing and packaging of genomic viral DNA, as well as production of precursors for DNA synthesis (eg., alkaline deoxyribonuclease: Weller *et al*, 1990).

The detailed mechanism by which HSV DNA replicates is not fully understood. The viral genome contains three origins of replication, one in U_L (Ori_L) and one in each R_S component (Ori_S) (reviewed by Hammerschmidt and Sugden, 1990). Deletion analysis has shown that one copy of an Ori sequence may suffice for viral DNA replication (Igarashi *et al*, 1993). Sedimentation studies and restriction endonuclease analysis have shown that high molecular weight "endless" viral DNA molecules are generated during HSV DNA replication (Ben-Porat *et al*, 1977; Jacob and Roizman, 1977; Jacob *et al*, 1979; Jongeneel and Bachenheimer, 1981). During infection in cell culture the termini of input viral genomes are observed to fuse soon after entry into cells, generating circular molecules (Poffenberger and Roizman, 1985). It has been proposed that these circular HSV genomes serve as templates for synthesis of concatemeric DNA by a "rolling circle" mechanism (Poffenberger and Roizman, 1985; Garber *et al*, 1993).

Unit length genomes are thought to be cleaved from these concatemeric intermediates (Jacob *et al*, 1979; Vlazny *et al*, 1982) and packaged into preformed capsids. A feature of

HSV DNA replication is inversion of the L and S components, generating four isoforms in equimolar amounts (Hayward *et al*, 1975; Delius and Clements, 1976; Clements *et al*, 1976). It is envisaged that alternate cleavage of concatemeric intermediates, generated from a single monomeric template, could account for the generation of two isoforms. The remaining two isoforms are thought to be generated by homologous recombination, and the repeated *a* sequences appear to play a role in recombination-mediated segment inversion (Mocarski *et al*, 1980; Smiley *et al*, 1981; Mocarski and Roizman, 1982; Bruckner *et al*, 1992; Dutch *et al*, 1992).

The rolling circle model does not adequately explain some of the experimental observations of HSV DNA replication. First, the kinetics of rolling circle DNA synthesis would result in linear increase in DNA mass over time, with time of synthesis of one unit length genome estimated at 20-40 mins (Jacob and Roizman, 1977). However, such linear increase does not correlate with the rapid amplification (several hundred-fold) of HSV DNA observed over a few hours in infected cells. Second, it has been shown that replicative intermediates consist of concatemers in which the L segments of 50% of adjacent viral genomes lie in the opposite orientation (Zhang *et al*, 1994). It is unlikely that such molecules arise from a simple rolling circle replication strategy. Consequently it has been proposed that a plasmid-like amplification strategy, similar to that of the 2μ plasmid of yeast (Futcher, 1986) may have preceded rolling circle replication (Hammerschmidt and Sugden, 1990; Hammerschmidt and Mankertz, 1991; Zhang *et al*, 1994). Initial HSV DNA amplification by a mechanism similar to 2μ circle replication could, on one hand, provide for rapid increase in templates and on the other, generate dimeric circular molecules with L segments in opposing orientations, providing templates for subsequent rolling circle replication capable of generating head to tail concatemers from which the four isomeric forms might be cleaved.

Virion assembly and egress

Studies on PRV suggest that following replication of alphaherpesvirus DNA and expression of γ genes empty viral capsids accumulate in the infected cell nucleus (Ben-Porat and Kaplan, 1973). The mechanism whereby concatemeric viral DNA intermediates are cleaved into unit length genomes (Jacob *et al*, 1979; Vlazny *et al*, 1982) and packaged into preformed capsids is not fully elucidated. The processes of cleavage and encapsidation appear to be linked, requiring cleavage/packaging signals (pac 1 and pac 2) located within the *a* sequence (Varmuza and Smiley, 1985; Deiss and Frenkel, 1986; Deiss *et al*, 1986).

Late in infection the nuclear envelope becomes convoluted and thickens in patches (Nii *et al*, 1968). This is associated with the accumulation of viral structural proteins, envelope glycoproteins and capsid assembly. The route by which the capsids acquire tegument and an envelope is not yet defined. Stackpole (1969) originally proposed that capsids become enveloped at the inner nuclear lamellae, de-enveloped at the outer nuclear lamellae, re-enveloped by the endoplasmic reticulum and released into the extracellular environment either by envelopment at the plasma membrane or by fusion of vesicles carrying enveloped viruses at the plasma membrane. This is, at least in part, supported by electron micrographs of capsids apparently in the process of budding through the inner nuclear membrane and of enveloped virions between the lamellae of the nuclear envelope and within cytoplasmic vesicles (Nii *et al*, 1968; Schwartz and Roizman, 1969; Rixon *et al*, 1992). Alternatively, virions may be secreted via the Golgi apparatus following a pathway similar to that taken by secreted soluble proteins (Johnson and Spear, 1982). The complete replicative cycle of HSV in cultured cells takes approx. 18-20 hr (Darlington and Granoff, 1973). It is widely accepted that, at least in tissue culture systems, cells productively infected with HSV do not survive.

Effect of HSV infection on the host cell

HSV infection induces morphological changes in the host cell nucleus and extensive disruption of host macromolecular synthesis (reviewed by Fenwick, 1984). The earliest cytopathic effects involve the cell nucleus. Nucleoli initially enlarge then begin to disintegrate by 4 hr after infection (Scotto *et al*, 1979; Dargan, 1986). Coinciding with the ultrastructural changes observed in nucleoli, ribosomal RNA (rRNA) synthesis is inhibited, up to 90% by 4 hr after infection (Roizman, 1969).

During infection virus-induced alterations are observed in nuclear chromatin distribution (Scotto *et al*, 1979; Martin *et al*, 1987) and in chromatin condensation (Hampar and Ellison, 1961; Stich *et al*, 1964; Peat and Stanley, 1986). Within 3 hr of infection despiralization of specific chromatin regions is observed, most notably in region q21 of chromosome 1 (1q21, Peat and Stanley, 1986) which encompasses the genomic location of the class 1 U1 small nuclear RNA (snRNA) pseudogenes (Lindgren *et al*, 1985). Consequently, it has been proposed that the uncoiling events observed reflect increased transcription of various host genes immediately following viral infection (Peat and Stanley, 1986). It has been shown that HSV-induced chromatid despiralization is dependent on expression of α, but not β or γ genes (Peat and Stanley, 1986). Virus-induced chromosomal damage, including extensive non-specific chromatid breakage, increases as infection progresses (Stich *et al*, 1964; Peat and Stanley, 1986).

During HSV infection virus-induced inhibition of host macromolecular synthesis is observed (reviewed by Fenwick, 1984). This inhibition displays two phases (1) a primary shutoff instigated by a virion component, *vhs*, which induces rapid degradation of mRNAs and the concomitant suppression of most cellular protein synthesis (Fenwick and Walker, 1978; Strom and Frenkel, 1987; Kwong *et al*, 1988; Becker *et al*, 1993) and, (2) a

secondary shutoff, which reduces the remaining levels of host protein synthesis and requires HSV gene expression (Nishioka and Silverstein, 1978; Fenwick and Clarke, 1982; Read and Frenkel, 1983).

HSV infection has a dramatic effect on the subnuclear distribution of the cellular small nuclear ribonucleoprotein (snRNP) complexes involved in pre-mRNA splicing (Martin *et al*, 1987; Bachman *et al*, 1989; Phelan *et al*, 1993). HSV-induced snRNP redistribution is mediated by ICP27 (Phelan *et al*, 1993) and resembles the alteration of snRNP distribution in uninfected cells when transcription is inhibited (Carmo-Fonseca *et al*, 1991b). snRNP redistribution in HSV infected cells is observed by 2 hr after infection and may play an integral role in the secondary inhibition of cellular mRNA expression (Phelan *et al*, 1993).

Cellular DNA synthesis is inhibited by up to 80% 2.5 hr after infection (Roizman, 1969). This suppression occurs in the absence of detectable viral protein synthesis and may be linked to the partial inhibition of cellular RNA synthesis (Fenwick and Walker, 1978). Viral DNA replication takes place in virus-induced nuclear compartments. As viral DNA replication is initiated, the intranuclear sites of DNA synthesis gradually shift from small dispersed foci (Huberman *et al*, 1973; Hozák *et al*, 1993) to globular structures which contain several virally encoded proteins essential for HSV DNA replication (Quinlan *et al*, 1984). The globular structures increase in size as DNA replication progresses (Rixon *et al*, 1983; Randall and Dinwoodie, 1986; de Bryn Kops and Knipe, 1988) and the number in each cell is dependent on the multiplicity of infection (Randall and Dinwoodie, 1986). Further, replication of virus in particular areas of the nucleus appears to induce physical redistribution of host cell DNA (Randall and Dinwoodie, 1986).

1.3 PATHOGENESIS OF HSV

1.3.1 Clinical significance

Herpes simplex affects millions of people worldwide with clinical manifestations ranging from mild vesicular lesions of the skin and mucous membranes in the majority of cases to more severe, sometimes fatal disease in a minority of individuals (Nahmias and Roizman, 1973; Corey and Spear, 1986; Whitley, 1990).

Problematic HSV infections include keratoconjunctivitis (ocular infection) which may cause corneal scarring. Indeed HSV is the most common cause of unilateral blindness in the USA (Kaufman, 1978; Pavan-Langston, 1984).

HSV acquired *in utero* or at birth can result in blindness, mental retardation and potentially lethal disseminated infection (Stagno and Whitley, 1985). HSV infection of immunocompromised individuals may result in locally aggressive peripheral lesions which may take weeks to heal (Corey and Spear, 1986). Rarely, disseminated infection involving many organs of the body may occur in immunocompetent adults and is potentially lethal if untreated (Whittaker and Hardson, 1978; Whorton *et al*, 1983). HSV induced encephalitis may result from primary or recurrent infection usually causing devastating, and frequently lethal, neurological damage (Whitley *et al*, 1982; Corey and Spear, 1986).

1.3.2 Primary productive infection

HSV is transmitted by direct contact and primary productive infection typically involves skin or mucous membranes. Virus replicates at the portal of entry in cells of ectodermal

origin, much as described for infection of cells *in vitro*. Infected epidermal cells undergo characteristic morphological changes including formation of syncytia, condensation of nuclear chromatin, nuclear degeneration and cell death (Wagner and Roizman, 1969; Darlington and Granoff, 1973). Viral replication is accompanied by tissue necrosis and infiltration of inflammatory cells and localized vesicles containing virus, cellular debris and inflammatory cells may form in the upper layers of the epidermis (Fleming, 1992). Vesicles, initially containing clear fluid and large amounts of infectious virus, become pustular as the lesion develops with concomitant loss of infectious virus. Primary herpetic lesions may take two to three weeks to heal completely and are not commonly associated with scarring (Buddingh, 1953). During primary peripheral infection, virus enters local sensory nerve endings and travels within axons to the neuronal cell bodies located in sensory ganglia (Cook and Stevens, 1973). Neuronal infection, unlike infection of epithelial cells, has two possible outcomes namely, (i) viral replication generating infectious progeny or, (ii) latency.

Primary oral infection is usually asymptomatic. Among symptomatic infections, the most common disease is gingivostomatitis, which is the classical presentation of HSV infection in young children (Whitley, 1990). Mouth and gums become covered in vesicles which rupture and ulcerate as the disease progresses. Rarely, more severe forms of infection occur, including the development of zosteriform-like lesions involving the entire dermatome (Mok, 1971; Music *et al*, 1971), potentially fatal disseminated neonatal herpes and meningoencephalitis. Like primary oral infections, primary genital infection may be asymptomatic; about 30% are clinically apparent (Mertz *et al*, 1992), associated for instance, with painful blistering of genital skin and mucous membranes.

1.3.3 Reactivation and recrudescence

Recurrent HSV infection, which may develop despite the presence of circulating neutralizing antibodies (Burnet and Williams, 1939), arises through reactivation of latent virus harboured in sensory ganglia, rather than by reinfection. The classical form of recurrent infection is a crop of vesicles around the mucocutaneous junctions of the lips or nose. The disease is known variously as a cold sore, a fever blister, herpes simplex, herpes labialis, herpes facialis or herpes febrilis and is most commonly associated with HSV-1. Latent virus may reactivate spontaneously or in response to stimuli such as sunburn, menstruation and stress (Nahmias and Roizman, 1973). How such stimuli are manifest at the molecular level is not known. As a consequence of reactivation, infectious virus is generated and transported via nerves, to the skin. Reactivation may result in the generation of a clinically apparent skin lesion (recrudescence) or virus may be shed asymptomatically (recurrence) (Wildy *et al*, 1982). Asymptomatic shedding is an important mode of transmission of herpes simplex (Mertz *et al*, 1992).

1.4 HSV LATENT INFECTION

Latency is a central feature of HSV infection. The molecular mechanism for the establishment and maintenance of latent infection is not known. Much of our knowledge regarding latent infection has arisen from studies using experimentally infected animals.

1.4.1 Animal models of latency

To be useful in the study of latency models should establish latency at high and consistent incidence in predicted tissues, preferably mimicking infection in humans with respect to

pathogenesis and the molecular level. Numerous animal systems have been developed and, although none can fully reproduce the virus-human host interaction, particularly with respect to recurrent infection, models have proven useful for the analysis of specific aspects of HSV latent infection.

Currently, the animal models most frequently used to study latency are mouse and rabbit models for HSV-1 and guinea pig models for HSV-2.

Mouse models

The size, cost and availability of numerous defined inbred strains are obvious advantages of the murine models. Moreover, HSV latency in mice is stable ie., spontaneous reactivation is rare. In mouse models, eyes, ears, footpads and flanks are the most common peripheral sites of inoculation. Typically, virus replicates at the site of inoculation and, usually within one week, viral antigens, viral DNA and infectious virus may be detected in ganglia innervating the inoculation site (Stevens and Cook, 1971; Hill *et al*, 1975; Simmons and Nash, 1984). After recovery from primary infection infectious virus, viral antigens and viral mRNAs cannot be detected in the ganglia. However, infectious virus can be recovered by culturing ganglia *in vitro* (explant culture) for several days (Stevens and Cook, 1971). This provides an experimental definition for latent virus that is, virus that does not exist in an infectious form yet may be induced to reactivate and produce infectious progeny virions (Roizman and Sears, 1987).

Rabbit models

In rabbits the most commonly used route of inoculation is corneal scarification which leads to establishment of latent infection in trigeminal ganglia (Stanberry, 1992). Recurrent infections in rabbits can be spontaneous or induced by a variety of stimuli. Like recurrent

infection in humans virus may be shed asymptomatically ie., clinical symptoms do not always follow reactivations (Nesburn *et al*, 1977; Berman and Hill, 1985; Hill *et al*, 1985; Gordon *et al*, 1990). Thus the rabbit is an amenable system in which to investigate reactivation of infection.

Guinea pig models

Guinea pigs are generally used as models in the investigation of genital herpes. Following intravaginal inoculation with HSV-2, guinea pigs develop a self-limiting infection (reviewed by Stanberry, 1986) which shares many features with genital infections in humans, including the establishment of latent infection in sacral dorsal root ganglia (drg) (Scriba and Tatzber, 1981; Stanberry *et al*, 1982) and frequent, spontaneous reactivations. Although valuable for investigation of recurrent infections, the high frequency of spontaneous reactivation in this model is a disadvantage for the study of latency.

1.4.2 Models of latency *in vitro*

Latently infected neurons constitute only a small proportion of the cells in ganglionic tissues rendering the analysis of latency in intact animals expensive and difficult. *In vivo* studies are further complicated by the heterogeneity of neural tissue and the complexity of the host environment. For these reasons many workers have attempted to establish *in vitro* latency systems.

Inhibition of productive infection in non-neuronal cells

Attempts to establish latent infection in permissive cells of non-neuronal origin have relied upon the use of various inhibitors of viral growth (eg., Wigdahl *et al*, 1981; Shiraki and Rapp, 1986) and/or supraoptimal temperatures (Russell and Preston, 1986) to arrest viral

replication. Such approaches have enabled the establishment of long-term persistent infections, with low levels of virus production which can be reactivated by various treatments (Wigdahl *et al*, 1981; Sheck *et al*, 1986; Russell and Preston, 1986). The main criticism of this type of system is that suppression of viral replication in cultured cells may not reflect latent infection in neurons (Roizman and Sears, 1987). Indeed, in several instances where the molecular characteristics of *in vitro* latent infections have been compared with the known characteristics of latency *in vivo*, differences have been found, for instance, expression of ICP0 and ICP8 in latently infected cultured cells was reported by Shiraki and Rapp (1989). Further, viral DNA may persist in a unit length linear form (Wigdahl *et al*, 1984) or in a hypermethylated state (Youssoufian *et al*, 1982). Neither of these findings are characteristic of latent viral DNA in neurons *in vivo*.

Infection of primary neuron cultures and immortal cells of neuronal origin

In attempts to establish *in vitro* latency systems that more closely mimic latency *in vivo*, several groups have resorted to infection of primary sensory neurons in the presence of acyclovir (eg., Wilcox and Johnson, 1988) or infection of non-permissive immortalized cells of neuronal origin (Nilheden *et al*, 1985; Wheatley *et al*, 1990). Some aspects of latency in these models resemble latent infection of neurons *in vivo*. For instance, latent infection *in vitro* can be established without immediate early gene expression (Wilcox and Johnson, 1988; Wheatley *et al*, 1990) and further, the synthesis of viral transcripts similar to those found in latently infected ganglia has been reported (Wheatley *et al*, 1990; Doerig *et al*, 1991). Thus neuronal cell models may prove to be the most appropriate of the currently available *in vitro* systems for analysis of latent infection.

1.4.3 Site of latent infection

HSV establishes latent infections in neurons of sensory ganglia (Cook *et al*, 1974; Cook and Stevens, 1976; McLennan and Darby, 1980). The involvement of neural tissues in persistence of HSV was first suggested by the observation that facial herpetic lesions often followed surgical trauma to the trigeminal ganglion (Cushing, 1905). HSV neurotropism was demonstrated subsequently in animals (Teague and Goodpasture, 1923). These observations led to the proposal that following a primary infection HSV remains latent within ganglia after the local lesion has healed and that, neural disturbances are frequently the basis of subsequent outbreaks (Goodpasture, 1929). This hypothesis was proven with the advent of ganglion explant culture methods which showed that infectious virus could be recovered from sensory ganglia of latently infected experimental animals (Stevens and Cook, 1971; Stevens *et al*, 1972; Scriba, 1975) and human cadavers (Bastian *et al*, 1972; Baringer and Swoveland, 1973). HSV has also been isolated from tissue containing neurons including autonomic ganglia (Price *et al*, 1975; Martin *et al*, 1977) and brains of experimentally infected animals (Knotts *et al*, 1973; Cook and Stevens, 1976; Cabrera *et al*, 1980; Rock and Fraser, 1983), although the prevalence of latency is much lower in the central nervous system (CNS) than in sensory ganglia (Tullo *et al*, 1982; Hill *et al*, 1983). Latent virus has not been recovered from human CNS despite the detection of DNA sequences homologous to the HSV genome in human brain (Fraser *et al*, 1981). The low incidence of latency in CNS may reflect ineffective reactivation of latent virus during explant culture.

During reactivation the incorporation of thymidine into viral DNA, viral antigens and virus particles are detected in neurons before other ganglionic cell types (Cook *et al*, 1974). Further, in analysis of a range of tissues derived from experimentally infected mice

following recovery from systemic HSV infection, Cook and Stevens (1976) found that virus was recoverable only from tissues containing neurons. Reactivation analysis of ganglionic tissues latently infected with HSV-1 temperature-sensitive mutants showed that, following reactivation at temperatures non-permissive for viral replication, viral antigens were detected exclusively in neurons (McLennan and Darby, 1980). Moreover, limited viral transcription has been observed in sensory neurons of latently infected ganglia using *in situ* hybridization (Deatly *et al*, 1987; Stevens *et al*, 1987; see also section 1.4.5). These observations overwhelmingly suggest that virus persists primarily, if not exclusively, in neurons.

1.4.4 Latent viral DNA

Physical characteristics

HSV-1 genomes that persist in latently infected animals and humans lack detectable termini (Rock and Fraser, 1983; Efstathiou *et al*, 1986). Such 'endless' viral DNA is consistent with circularization, concatenation or concatemerization of genomes or, integration into host cell chromatin. Latent viral DNA is nucleosome associated (Deshmane and Fraser, 1989) and may exist as unintegrated episomes as shown by buoyant density gradient centrifugation analysis of latently infected brain stem DNA (Mellerick and Fraser, 1987).

Abundance in latently infected tissues

The number of HSV genomes present in latently infected cells is still in question. To date, HSV DNA has not been detected directly by hybridization *in situ,* thus direct estimation of DNA copy number has not been possible. Early DNA analyses suggested that large amounts of viral DNA were present in latently infected tissues (Rock and Fraser, 1983;

Efstathiou *et al*, 1986). Typically, approximately 1% of neurons in ganglia appear to be latently infected, as estimated by reactivation studies on dissociated latent ganglia (Walz *et al*, 1976; Nicholls *et al*, 1989) or *in situ* hybridization detection of viral latency associated transcripts (eg., Tenser *et al*, 1989). By comparing the amounts of HSV DNA extracted from latently infected tissues with estimates of the number of latently infected neurons in corresponding material, the estimated numbers of HSV genomes per cell range from 20 (eg., Stevens, 1989; Rødahl and Stevens, 1992; Simmons *et al*, 1992) to several hundred (Roizman and Sears, 1990; Simmons *et al*, 1992). The presence of a large number of viral genomes in a latently infected neuron would suggest that viral DNA can be amplified at some stage during the establishment and or, maintenance of latent infection. Paradoxically, it has been shown that (i) replication-defective viral mutants can still establish latency (Lofgren *et al*, 1977; McLennan and Darby, 1980; Watson *et al*, 1980; Efstathiou *et al*, 1989; Leib *et al*, 1989a) and (ii), latency may be established at sites which do not show expression of viral proteins at any stage during primary infection (Speck and Simmons, 1992). Establishment of latency without detectable viral gene expression suggests that amplification of HSV DNA by virally encoded enzymes is not an essential component of the establishment process. In support of this conclusion, very little HSV DNA can be recovered from ganglia of animals latently infected with replication defective viral mutants (Efstathiou *et al*, 1989).

In studies utilizing viral spread in the nervous system Simmons *et al* (1992) demonstrated two patterns of HSV DNA persistence in ganglia. First, ganglia innervating the peripheral site of inoculation, in which viral protein expression was detected during primary infection, displayed high genome copy latency (upwards of 200 HSV genomes per latently infected neuron). Second, a low copy latency (approx 20 HSV genome copies per infected neuron) was associated with ganglia which did not innervate the inoculation site or show signs of

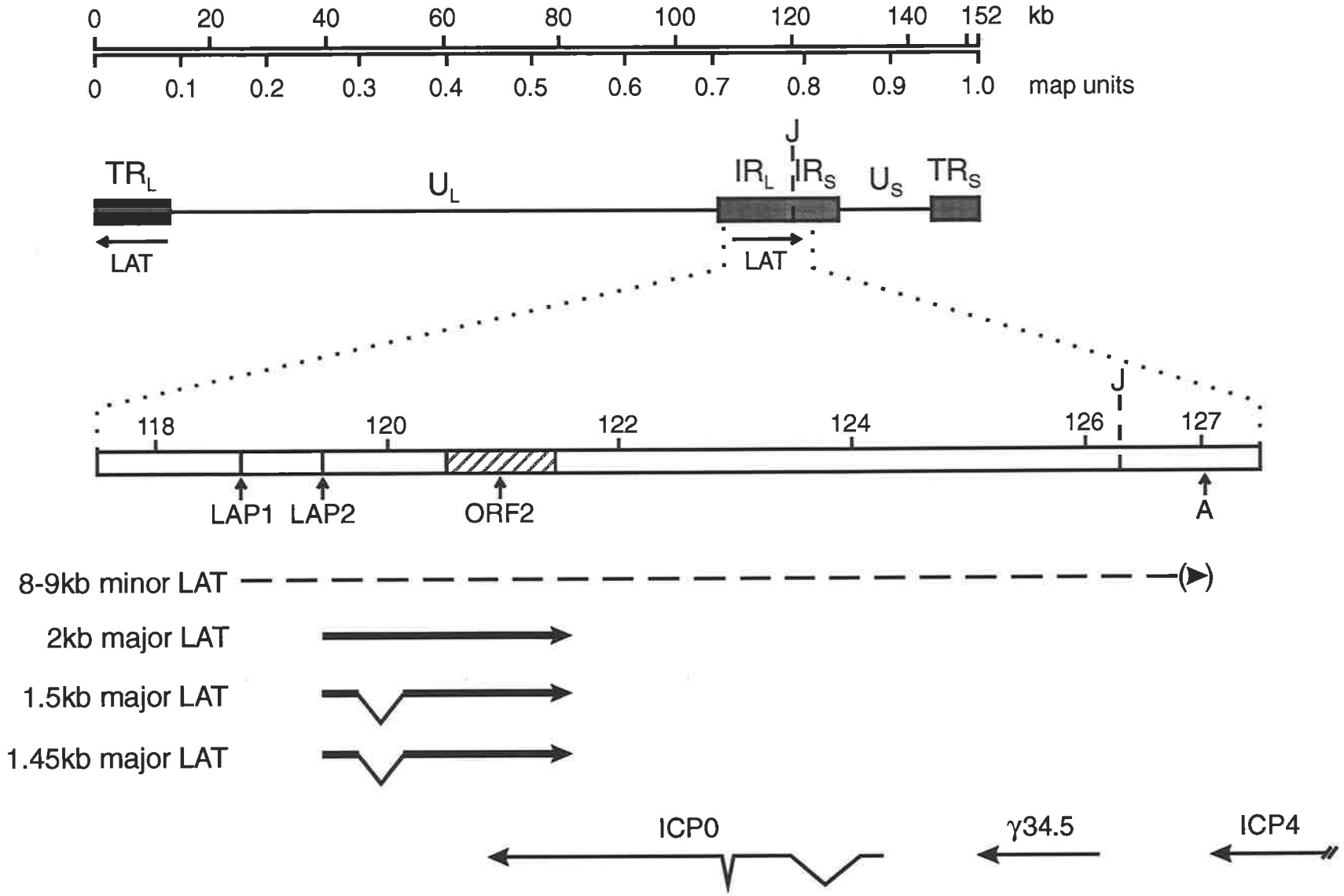
viral antigen production during primary infection. It is interesting to note that, in this instance, the bulk of the latent viral DNA did not correlate with the majority of reactivation-competent latent neurons.

Given that, at least in cell culture, viral replication is invariably cytolytic (Roizman and Sears, 1990) it is difficult to conceive that multiple copy latency may arise through productive infection. There are a number of other possible explanations. First, estimates of the number of latent sites may be too low. Second, many viral genomes may enter the neuron during the establishment of latency. Third, HSV DNA may be amplified as a result of abortive infection (Simmons and Tscharke, 1992) or through host cell enzymes (Sears and Roizman, 1990). Fourth, the bulk of the HSV DNA persisting in ganglia may be in a reactivation-defective, transcriptionally inactive form, not necessarily in neurons.

1.4.5 HSV-1 gene expression during latency

Early reports that selected viral proteins may be expressed during latent infection of neurons (Green *et al*, 1981 and 1984) have not been confirmed (reviewed by Fraser *et al*, 1992 and Stevens, 1989). During latency most of the viral genome appears to be transcriptionally inactive. In 1987 two independent *in situ* hybridization studies of latently infected murine ganglia detected transcripts from a limited region of the viral genome (Deatly *et al*; Stevens *et al*). These RNAs, observed only in the nuclei of neurons, mapped to the L repeats and were shown to partially overlap the gene encoding ICP0, being complementary to the ICP0 RNA (Stevens *et al*, 1987; Spivack and Fraser, 1987) (Figure 1.1). Subsequently, these apparently abundant latency associated transcripts (major LATs) were detected in latently infected ganglia of humans (Croen *et al*, 1987; Gordon *et al*, 1988; Krause *et al*, 1988; Steiner *et al*, 1988; Stevens *et al*, 1988; Wechsler *et al*, 1988a)

Figure 1.1. Map of the HSV-1 genome structure illustrating the regions from which LATs are transcribed. The expanded view of the internal repeat region (from 118 to 127 kb) shows the relative positions of LAT promoter regions (LAP1 and LAP2), conserved open reading frame 2 (ORF2, hatched box) and the predicted polyadenylation signal (A) for the putative 8 to 9 kb minor LAT (broken line). The positions of the 1.45, 1.5 and 2 kb major LATs (thick arrows) and the transcripts coding for ICPO, γ34.5 and part of ICP4 (thin arrows) are depicted. Also indicated are the long unique segment (U_L), short unique segment (U_S), the terminal repeat sequences flanking U_L (TR_L) and U_S (TR_S), the internal repeat sequences flanking U_L (IR_L) and U_S (IR_S) and the junction between IR_L and IR_S (J).


0 20 40 60 80 100 120 140 152 kb
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 map units
J
TRL
UL
IRL IRS
US
TRS
LAT
LAT
J
118 120 122 124 126 127
LAP1 LAP2
ORF2
A
8-9kb minor LAT
2kb major LAT
1.5kb major LAT
1.45kb major LAT
ICP0
γ34.5
ICP4

and rabbits (Rock *et al*, 1987b; Wagner *et al*, 1988b).

Northern (RNA) blot analysis of latently infected ganglia indicates that major LATs are comprised of up to three colinear transcripts ~2 kb, ~1.5 kb and ~1.45 kb in length (Spivack and Fraser, 1987). Related LATs have been identified during latent infections with other α-herpesviruses such as HSV-2 (Suzak and Martin, 1989; Mitchell *et al*, 1990a), BHV-1 (Rock *et al*, 1987a; Kutish *et al*, 1990) and PRV (Rock *et al*, 1988; Cheung, 1989; Priola *et al*, 1990).

It has been estimated that each neuron latently infected with HSV-1 contains between 20,000 and 50,000 LATs, the 2 kb species constituting 50-90% of the LATs, depending on the strain of HSV-1 (Wagner *et al*, 1988 a&b). The 2 kb and 1.5 kb LATs share 5' and 3' termini and appear to differ only by the excision of an ~0.5 kb intron from the larger species (Wagner *et al*, 1988 a&b; Wechsler *et al*, 1988a; Spivack *et al*, 1991). It is not clear how the 1.45 kb LAT is generated. It has been suggested that major LATs may arise through splicing of a larger, less abundant primary transcript (Dobson *et al*, 1989; Farrell *et al*, 1991; Spivack *et al*, 1991). The shared 5' termini of major LATs resembles a splice donor signal (Dobson *et al*, 1989; Farrell *et al*, 1991; Spivack *et al*, 1991) and located approximately 2 kb downstream of this is a consensus splice acceptor site (Farrell *et al*, 1991). Consistent with the hypothesis that major LATs are introns is the observed nuclear localization (Deatly *et al*, 1987; Stevens *et al*, 1987) and the general lack of detectable polyadenylation of these RNAs (Wagner *et al*, 1988b). Furthermore, when the 2 kb LAT was cloned into a β-galactosidase gene, chimeric transcripts were efficiently spliced at the consensus splice donor and acceptor sites to generate translatable β-galactosidase mRNA. The excised 2 kb "intron" was more stable than either the primary or processed β-

galactosidase transcripts (Farrell *et al*, 1991). However, the use of these splicing sites in the generation of major LATs during latent infection is yet to be demonstrated.

Not all of the experimental findings are consistent with major LATs being introns. First, although there is some evidence for an 8-9 kb precursor LAT (see section on minor LATs below), spliced derivatives of the putative precursor RNA, which would be in the order of 6-7 kb, have not been detected. Second, sequence analysis of major LAT RNA PCR products and cDNA libraries from latently infected mouse ganglia, indicate that the 3' end of the 2 kb LAT may be located upstream of the consensus splice acceptor site (Spivack *et al*, 1991). Third, if the 2 kb major LAT is an intron it is unusual in that it has introns. Further, the 2 kb major LAT appears to be more stable than the primary transcript or the spliced product (Farrell *et al*, 1991; Spivack *et al*, 1991).

LAT promoter element

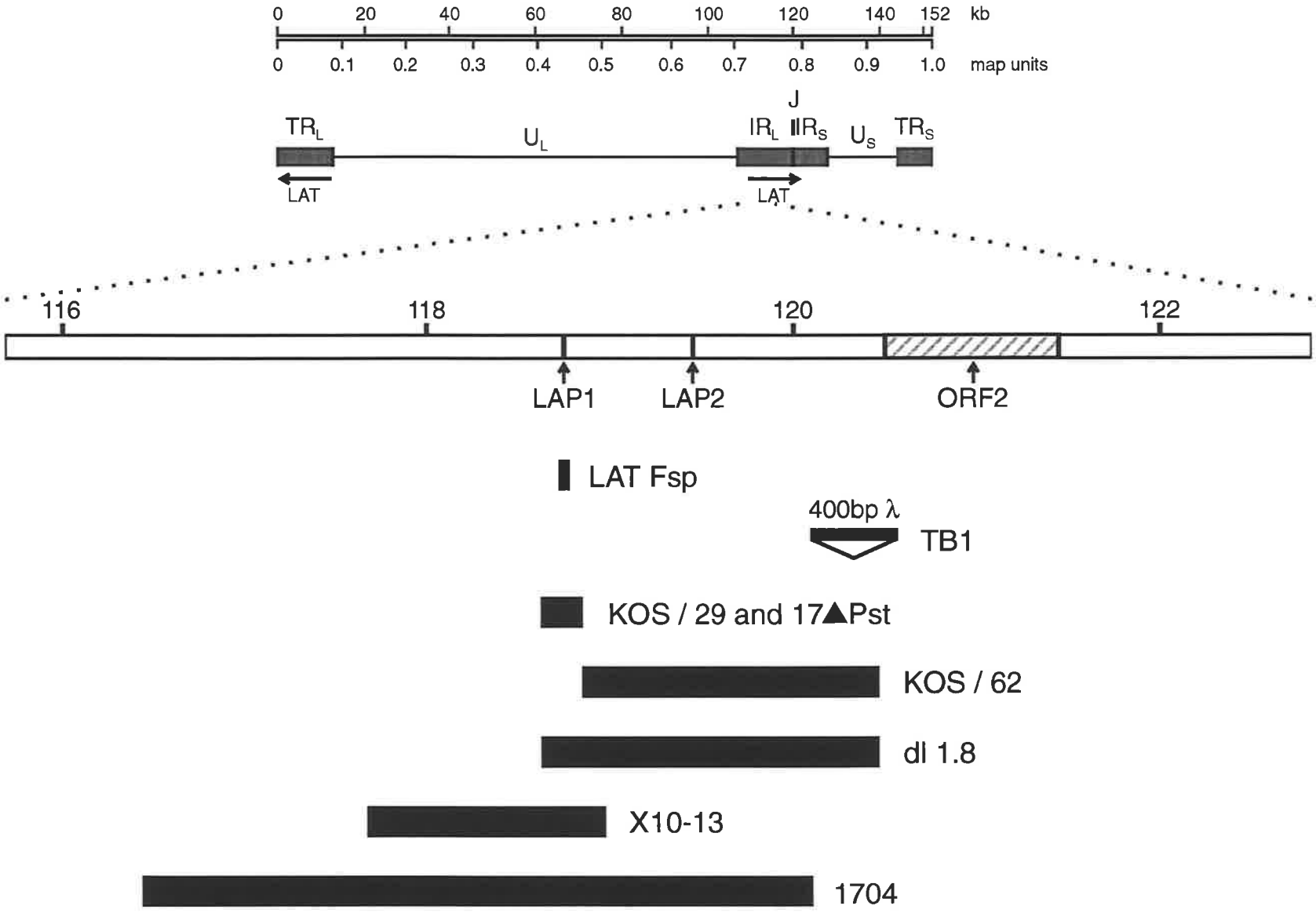
A promoter element, designated LAP1, apparently responsible for major LAT synthesis during latency is situated approximately 0.7 kb upstream of the shared 5' end of the major LATs (Figure 1.1; Dobson *et al*, 1989; Zwaagstra *et al*, 1989, 1990; Batchelor and O'Hare, 1990).

This region contains consensus sequences characteristic of an RNA polymerase II promoter including a TATA box, a potential CAAT box and Sp1-binding sites (Wechsler *et al*, 1988a, 1989). Other potential promoter regulatory regions include (i) a cyclic adenosine monophosphate (cAMP) responsive element binding (CREB, Leib *et al*, 1991) region, (ii) a palindromic sequence which binds an as yet uncharacterized host cell factor (ie., LAT promoter binding factor, LPBF) found in neuronal and non-neuronal cells (Zwaagstra *et al*, 1991) and (iii) sequences 0.79 to 1.27 kb upstream of the 5' end of major LATs which

enhance promoter activity in cells of neuronal origin (Batchelor and O'Hare, 1990, 1992; Zwaagstra *et al*, 1990, 1991). Further, Batchelor and O'Hare (1990) reported that LAT promoter activity is repressed by ICP4. This response is conferred by a 0.055 kb region downstream of the TATA box. Overall these regulatory sequences suggests that activity of LAP1 may be influenced by various host and viral factors.

HSV-1 mutants which have deletions encompassing LAP1 do not synthesize detectable amounts of major LATs during latent infection (see Figure 1.2; eg., Javier *et al*, 1988; Dobson *et al*, 1989; Leib *et al*, 1989b; Steiner *et al*, 1989; Block *et al*, 1993). These observations support the proposal that LAP1 is responsible for synthesis of LATs during latency. Two recent observations however, indicate that the control of transcription across the LAT region may be more complex than described above. First, Nicosia and co-workers (1993) report that two HSV-1 mutants, KOS/29 and 1704 which have deletions encompassing LAP1 in both copies of the LAT gene (Figure 1.2), synthesize a 2 kb LAT-related RNA during lytic infection and reactivation in ganglionic tissues of experimentally infected mice. This LAT-related RNA has extensive homology to the 2 kb major LAT. Transcription across the LAT region during latency however has not been demonstrated for either mutant (Dobson *et al*, 1989; Steiner *et al*, 1989). Second, sequences 0.19 kb to 0.59 kb upstream of the 5' end of major LATs can promote RNA synthesis with transcription initiating at or near the 5' end of the major LATs (Goins *et al*, 1994). This cryptic promoter region, designated LAP2, lacks a TATA box but contains several potential transcription regulatory elements including a sequence with homology to transcription-initiator elements present in some TATA-less promoters (Goins *et al*, 1994). It is possible that LAP1 and LAP2 are not independent promoters but rather components of a single complex promoter-regulatory region (Goins *et al*, 1994).

Figure 1.2 Schematic diagram illustrating the positions of mutations in the LAT mutant viruses described in the text (section 1.4.6). The relative positions in IR_L of sequences deleted from (solid boxes), or inserted in (triangle) the mutant viral genomes are illustrated. For all viruses represented the mutations are reproduced in TR_L (not illustrated). The positions of the LAT promoter elements (LAP1, LAP2), ORF (hatched box) and the putative minor LAT polyadenylation site (A) are indicated.


0 20 40 60 80 100 120 140 152 kb
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 map units
J
TRL
UL
IRL
IRS
US
TRS
LAT
LAT
116
118
120
122
LAP1
LAP2
ORF2
LAT Fsp
400bp λ
TB1
KOS / 29 and 17▲Pst
KOS / 62
dl 1.8
X10-13
1704

Minor LATs

In situ hybridization analysis of latently infected tissues has detected transcripts of low abundance, designated minor LATs, arising from the region between LAT promoter and the 5' terminus of major LATs and also from sequences up to 8 kb downstream of the major LATs (Deatly *et al*, 1987, Rock *et al*, 1987b; Krause *et al*, 1988; Wagner *et al*, 1988a; Wechsler *et al*, 1988b; Dobson *et al*, 1989; Mitchell *et al*, 1990b; Zwaagstra *et al*, 1990).

Consequently it has been suggested that minor LATs represent a single RNA species extending for 8-10 kb downstream of LAP1 (Figure 1.1) and that the 2 kb and 1.5 kb LATs major LATs are generated through splicing of this RNA (reviewed by Fraser *et al*, 1992). However, analysis of the physical nature of these molecules has proved problematic because their abundance in latently infected tissues is below the level of sensitivity of Northern blotting. An 8-9 kb poly(A)$^+$ RNA containing LAT sequences has been detected by Northern blot analysis of RNA isolated from productively infected tissue culture cells (Dobson *et al*, 1989). Zwaagstra *et al* (1990) detected an ~8 kb RNA containing minor LAT sequences on Northern blot analysis of ganglionic RNA isolated from rabbits latently infected with HSV-1. The abundance of this RNA species was very low and it was only observed in a minority of latent RNA preparations (Zwaagstra *et al*, 1990). Given the frequency of spontaneous reactivation observed in rabbit models, it cannot be excluded that the ~8 kb transcript is a product of reactivation rather than latent infection.

Protein coding capacity of LATs

In their analysis of the minor LAT sequence between the proposed TATA box and the 5' terminus of the major LATs, McGeoch *et al* (1991) conclude that it is unlikely that this

minor LAT region encodes protein because, although the HSV-1 sequence contains three potential ATG translation initiation codons, HSV-2 possesses none and open reading frames are not conserved between the HSV-1 and HSV-2.

Major LATs display characteristics which are generally associated with introns, which does not favour a protein-coding role, with the caveat that protein expression from intronic RNA species has been noted in other systems (King and Piatigorsky, 1983; Perlam and Butow, 1989). Sequence analysis of the major LAT region has identified open reading frames (ORFs) (Wagner *et al*, 1988a; Wechsler *et al*, 1989) of which only ORF2 (Figure 1.1; Wagner *et al*, 1988a) shows significant sequence conservation between strains of HSV-1 and also between HSV-1 and HSV-2 (Wechsler *et al*, 1989; McGeoch *et al*, 1991). The significance this conservation is not known as ORF2 overlaps the 3' end of the ICP0 coding sequence which may, given the positional base preference in nucleotide composition (a characteristic of HSV-1 coding sequences: Perry and McGeoch, 1988), non-specifically enhance nucleotide sequence conservation. Moreover, ORF2 does not display such nucleotide bias and the HSV-2 counterpart of ORF2 is disrupted by two stop codons within the region overlapping the ICP0 coding sequence (McGeoch *et al*, 1991). Collectively these observations suggest that ORF2 may not be translated, at least in its entirety, during infection. Indeed, disruption of ORF2 by frames shift mutation does not significantly affect the ability of HSV-1 to establish latency or reactivate (Farrell *et al*, 1993).

Minor poly(A)$^+$ species of LAT-related transcripts have been observed (Wagner *et al*, 1989b; Dobson *et al*, 1989; Zwaagstra *et al*, 1990) and it is possible that during certain stages of the infectious cycle, protein products from the LAT region may be transiently expressed owing to alternate splicing events or multiple sites of transcription initiation

(Nicosia *et al*, 1993; Goins *et al*, 1994). Recently, Doerig *et al* (1991) expressed the 3' 112 codons of ORF2 in *Escherichia coli* as part of a tryptophan E (*trp*E) fusion protein and used the chimeric product to raise antibodies in rabbits. The resulting immune serum recognized an 80 kDa and a 45 kDa polypeptide. These antigens were not detected in mock-infected neuronal cells or in neurons latently infected *in vitro* with HSV-1 LAT null mutant *dl*1403 (Doerig *et al*, 1991). The abundance of the 45 kDa polypeptide varied between extracts suggesting that it may be a product of degradation of the 80 kDa protein (Doerig *et al*, 1991). The significance of these latency associated antigens (LAA) is difficult to ascertain for three reasons. First, the LAAs have not been further characterized, second, the LAAs are much larger than the ~33 kDa polypeptide predicted from the ORF2 sequence (Wechsler *et al*, 1988a) and last, LAAs are yet to be detected during latent infection *in vivo*.

1.4.6 Function of the LATs

The role of the LATs is yet to be defined. It is also, at this stage, unclear if the LATs function as structural or protein-coding RNAs. Given the abundance of major LATs in latently infected tissues, it is predicted that LATs participate in the establishment, maintenance or reactivation of latent infection. Much of the phenotypic analysis of this region has focused on the use of LAT mutant viruses.

Effect on establishment of latent infection

Initially the LATs were thought to function in establishing or maintaining the latent state of the virus. Given that the major LATs overlap and are complementary to the 3' end of ICP0 mRNA, Stevens *et al* (1987) postulated that the LATs may regulate the expression of ICP0 by an antisense-RNA interference mechanism. As discussed previously, ICP0 is a

transcriptional activator of all three classes of HSV genes, its effect being most important at low multiplicities of infection and during reactivation (Cai and Schaffer, 1989; Everett, 1989). Suppression of ICP0 expression might precipitate the establishment of latency or help maintain the latent state by prevention of reactivation. However, experimental evidence in support of these proposals has been difficult to obtain. For instance, viral strains with mutations that impair or delete the synthesis of LAT retain the ability to establish stable latent infection, as assessed by the presence of reactivatable, latent viral DNA (Javier *et al*, 1988; Ho and Mocarski, 1989; Leib *et al*, 1989b; Sedarati *et al*, 1989; Steiner *et al*, 1989). However, it has been demonstrated recently that transcription of LATs may, at certain anatomical sites, play an important role in the establishment of latency (Sawtell and Thompson, 1992). In these experiments LAT deficient (LAT^-) mutants (KOS/29 and KOS/62; Figure 1.2), in comparison to wild type virus, established significantly lower levels of latency in trigeminal, but not dorsal root ganglionic neurons (Sawtell and Thompson, 1992).

Effect of LATs on reactivation

The effect of LAT expression on viral reactivation has been the focus of several recent studies. When the LAT promotor is intact, most LAT mutants reactivate with normal kinetics (Ho and Mocarski, 1989; Sedarati *et al*, 1989; Block *et al*, 1990). In contrast, viruses in which LAP1 has been mutated may show reduced levels of reactivation or delayed reactivation kinetics (Figure 1.2; Leib *et al*, 1989b; Hill *et al*, 1990; Trousdale *et al*, 1991; Block *et al*, 1993; Rader *et al*, 1993). The biological significance of altered reactivation observed for LAT mutant viruses is difficult to ascertain as it has been reported that particular mutant virus strains (X10-13, KOS/62 and 17▲Pst; Figure 1.2) display abberant reactivation from trigeminal, but not lumbrosacral, ganglia (Javier *et al*, 1989; Hill *et al*, 1989; Sawtell and Thompson, 1992; Devi-Rao *et al*, 1994). In comparison

to parental (wild type) virus strains KOS/62 and 17▲Pst viruses establish lower levels of latency in trigeminal, but not lumbrosacral, ganglia which may account for the reactivation phenotype in trigeminal ganglia.

Many physiological stimuli (eg., fever, sunburn, stress) or experimental stimuli (ultraviolet light, prostaglandins, epinephrine) that can trigger reactivation share the capacity to influence levels of intracellular cyclic nucleosides such as cyclic adenosine monophosphate (cAMP). Several studies have shown that agents which increase the levels of intracellular cAMP (eg., epinephrine) can accelerate reactivation of latent virus in explanted ganglia (Foster *et al*, 1989; Sainz de la Maza *et al*, 1989; Leib *et al*, 1991). Leib *et al* (1991) identified a cAMP responsive element (CRE) in the LAT promoter region. This CRE is sensitive to a variety of cAMP modulators and is responsible for the high basal activity of the LAT promoter in cells of neural origin (Leib *et al*, 1991). Furthermore, deletion of sequences spanning the LAT promoter, including the CRE, results in viruses (X10-13, 1704, 17▲Pst; Figure 1.2) which are impaired for epinephrine-induced, but not spontaneous, viral reactivation (Hill *et al*, 1990; Trousdale *et al*, 1991; Bloom *et al*, 1994). However, Rader *et al*, (1993) in analysis of LATFsp, a CRE mutant virus, and *dl*LAT1.8, a virus in which 1.8 kb of sequence spanning the LAT promoter has been deleted (Figure 1.2), have shown that, although LATFsp and *dl*LAT1.8 are reproducibly delayed in their reactivation kinetics relative to marker-rescued viruses, both show epinephrine-induced acceleration of viral reactivation. Thus the elements that are largely responsible for cAMP-mediated acceleration of reactivation lie outside the region deleted in *dl*LAT1.8, indicating that this response does not rely on the CRE element identified by Leib *et al* (1991).

How the LATs influence reactivation kinetics is not known. Block *et al* (1993) have shown that although, total virus yield is similar to that of wildtype virus, the titre of virus (TB1;

Figure 1.2) released from neurons during reactivation may be reduced for LAT-deficient mutants. This implies a possible role for LATs in the efficient egress of virus from neurons.

1.5 THE CELL NUCLEUS

A major requirement of the nucleus is packaging of the DNA genome in such a manner that it remains accessible for replication, selective transcription and accurate segregation during mitosis. An important component of nuclear organization is the nucleoskeleton, an insoluble proteinaceous matrix which is intimately associated with DNA organization and nuclear processes such as DNA replication and RNA synthesis (reviewed by Nelson *et al*, 1986). A consequence of the higher order organization of the nucleus is the development of domains with distinct biochemical functions.

1.5.1 Nucleolus

The most obvious example of the relationship between biochemical function and spatial distribution is the nucleolus, a distinct structural entity within which active ribosomal genes are sequestered from the rest of the genome and nucleoplasm. Nucleoli are the site of synthesis and processing of ribosomal RNA (rRNA) and also the assembly of preribosomal particles (for review see Hadjiolov, 1985).

1.5.2 Nuclear bodies

Several other subnuclear structures of unknown function, collectively termed nuclear bodies, have been identified on electron microscopic examination of nuclei (for review see

Padykula and Clark, 1981). The most extensively studied nuclear body is the coiled body which morphologically resembles a ball of coiled threads (Monneron and Bernhard, 1969). Coiled bodies are generally round and measure 0.5 to 1 μm in diameter. Frequently, but not exclusively, they are associated with the nucleolar periphery. Coiled bodies increase in size and number in response to increased levels of cellular gene expression (Lafarga *et al*, 1991; Brasch and Ochs, 1992; Spector *et al*, 1992; Carmo-Fonseca *et al*, 1992). Recent studies on the distribution of the small nuclear ribonucleoprotein (snRNP) complexes associated with splicing have demonstrated the presence of snRNPs in coiled bodies (Ráska *et al*, 1991; Carmo-Fonseca *et al*, 1991 a&b, 1992). Furthermore the association of snRNPs with coiled bodies is dynamic, being dependent on continuing RNA transcription (Spector *et al*, 1991; Carmo-Fonseca *et al*, 1992). The reason for the association between snRNPs and coiled bodies is not clear, although it has been proposed that coiled bodies are sites of recycling of snRNPs from post-splicing complexes (Lamond and Carmo-Fonseca, 1993).

1.5.3 Distribution of chromatin

At interphase eukaryotic chromatin consists of highly condensed, transcriptionally inactive heterochromatin and less condensed, generally transcriptionally active euchromatin. Heterochromatin is commonly located in an irregular band around the nuclear periphery, at the nucleolar surface and also in patches throughout the nucleoplasm. The distribution of specific transcriptionally active genes also appears to be non-random. For example, in Namalwa cells two Epstein-Barr virus genomes which are integrated into chromosome 1, is reproducibly localized to the inner 50% of the nuclear volume (Lawrence *et al*, 1988).

Replication of DNA occurs at hundreds of discrete sites in the nucleus (Nakayasu and

Berezney, 1989) and appears be co-ordinately regulated such that specific regions of the DNA are replicated at defined times in defined domains of the nucleus (reviewed by Spector, 1993).

1.5.4 Distribution of factors associated with RNA biogenesis

Transcription and splicing associated factors

Analysis of the distribution of newly synthesized RNA indicates that the transcription of messenger RNA (mRNA) occurs within hundreds of small domains scattered throughout the nucleoplasm (Jackson *et al*, 1993; Wansink *et al*, 1993). Concomitant with, or shortly after transcription, pre-mRNAs are polyadenylated and spliced to remove non-coding intron sequences. Splicing occurs in a multicomponent complex (spliceosome) containing a discrete class of small nuclear RNAs (snRNAs) complexed with defined protein factors (ie., small nuclear ribonucleoproteins, snRNPs) (reviewed by Zieve and Sauterer, 1990).

Immunofluorescent detection of protein or RNA components of snRNPs using human autoantibodies, or *in situ* hybridization detection of snRNAs, have shown these components to be concentrated in 20-50 nuclear speckles in addition to being distributed diffusely throughout the nucleoplasm (Spector *et al*, 1983; Nyman *et al*, 1986; Carmo-Fonseca *et al*, 1991 a&b; Huang and Spector, 1991; Spector *et al*, 1992). At the electron microscope level the speckles of splicing factors correspond to nuclear regions enriched in perichromatin fibrils and interchromatin granules (Fakan *et al*, 1984; Spector *et al*, 1991). In addition to the speckled distribution, several studies have demonstrated the snRNPs concentrated in coiled bodies in a proportion of cells in a given population (Carmo-Fonseca *et al*, 1991 a&b, 1992; Ráska *et al*, 1991; Spector *et al*, 1992).

Poly(A)$^+$ RNA

Carter *et al*, (1991) used fluorescence *in situ* hybridization (FISH) to examine the intranuclear distribution of poly(A)$^+$ RNA in cells at interphase. Poly(A)$^+$ sequences in this study found in 20-30 nuclear speckles which colocalized with snRNP domains. Further, the majority of poly(A)$^+$ speckles did not contact the nuclear envelope. These speckles, designated transcript domains, encompass interchromatin granules and perichromatin fibrils and consist of nascent and stable poly(A)$^+$ RNA (Carter *et al*, 1991). Fluorescently-tagged, β-globin pre-mRNA adopts a speckled distribution, coincident with the speckled pattern enriched in splicing factors (Wang *et al*, 1991), when microinjected into interphase nuclei. In contrast, microinjection of transcripts lacking introns produced a diffuse staining pattern indicating that intron sequences may confer upon pre-mRNA molecules the ability to associate with nuclear domains containing splicing factors (Wang *et al*, 1991).

By what path mature poly(A)$^+$ mRNAs migrate to the cytoplasm is yet to be determined. In order to gain insight to the movement of mRNA in the nucleus several groups have characterized the distribution of specific mRNA species.

Nuclear mRNA "tracks"

Lawrence and colleagues (1989) used FISH to analyse the distribution of EBV RNAs in Namalwa cells which contain two copies of the EBV genome closely integrated on chromosome 1 (Lawrence *et al*, 1988). In this study EBV RNA was detected as a curvilinear "track" of, on average, 5 μm length. These "tracks" extended between the nuclear interior and the periphery with 15% apparently in direct contact with the nuclear envelope (Lawrence *et al*, 1989). Subsequently nuclear RNA "tracks" have been reported for c-fos (Huang and Spector, 1991) and fibronectin (Xing *et al*, 1993) transcripts. On electron microscope examination c-fos "tracks" appear to leave the nucleus at discrete

points on the nuclear envelope (Huang and Spector, 1991). Using two colour FISH Xing *et al* (1993) demonstrated that in 88% of fibronectin tracks the gene was clearly positioned at one end of the track and further, that intron sequences were generally confined to a smaller part of the track than exons. Indeed the majority of fibronectin and c-fos RNA tracks are closely associated with domains enriched in splicing factors (Huang and Spector, 1991; Xing *et al*, 1993). Not all RNAs studied however display such RNA "tracks". For example, FISH for neurotensin RNA in neuroblastoma cells detects one or two intense foci of hybridization (Xing *et al*, 1993). Interestingly, probes specific for neurotensin introns detect these foci and produce a less intense, slightly punctate fluorescence dispersed throughout the nucleoplasm, excluding the nucleolus which is consistent with diffusion of intronic sequences following excision from pre-mRNA (Xing *et al*, 1993).

The observation of tracks of RNA extending from sites of transcription to discrete points at the nuclear envelope suggests a highly organized and regulated means of mRNA transport through the nucleoplasm to the nuclear pores, consistent with aspects of the "gene gating" hypothesis proposed by Blobel (1985).

1.6 AIMS OF THESIS

Current evidence suggests that cellular and viral RNAs are synthesized and processed by macromolecular complexes occupying discrete compartments within the nucleoplasm of a cell. Given that at the time of initiation of this project little was known about the generation and function of the HSV-1 LATs *in vivo*, the aims of this project were to (1) study the intranuclear topology of HSV-1 LATs in latently infected neurons *in vivo* and, (2) characterize the organization of macromolecular complexes associated with production of RNA in the nuclei of murine dorsal root ganglionic neurons with a view to gaining insight into the synthesis, processing and transport of LATs *in vivo*.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tissue culture cells

Vero and Neuro-2a cells were obtained from the American Type Tissue Culture Collection (ATCC, Rockville, Maryland, USA) and were free of mycoplasma. The Vero cell line (ATCC Cat# CCL 81) was initiated from the kidney of a normal, adult African green monkey by Y. Yasumura and Y. Kawakita (Chiba University, Chiba, Japan). The Neuro-2a cell line (ATCC Cat# CCL 131) was established from a spontaneous tumour of a strain A albino mouse by R.J. Klebe and F.H. Ruddle (Yale University, New Haven, Connecticut, USA). The Neuro-2a line consists of neuron-like cells and also small round stem cells.

Acetone-fixed HEp-2 cell monolayers on glass microscope slides were obtained from Immuno Concepts (Sacrimento, California, USA. Cat# SA 2013). The HEp-2 cell line (ATCC Cat# CCL 23) was established by A.E. Moore (Sloan-Kettering Institute, New York, New York, USA) from tumours that had been produced in irradiated-cortisonized rats after injection with human larynx epidermoid carcinoma tissue

2.1.2 Virus stocks

Experiments were done with cloned HSV-1 strain SC16, a clinical isolate of low passage history (Hill *et al*, 1975). The pathogenicity of strain SC16 has been studied for various routes of inoculation in 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). This virus is neurovirulent after cutaneous flank 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). Master and submaster stocks were stored in liquid nitrogen and fresh working stocks made at regular intervals from submaster stocks by infecting Vero cells at low multiplicity (0.1 plaque forming units (pfu) per cell). Aliquots of working stocks were stored at -70°C until required.

2.1.3 Mice

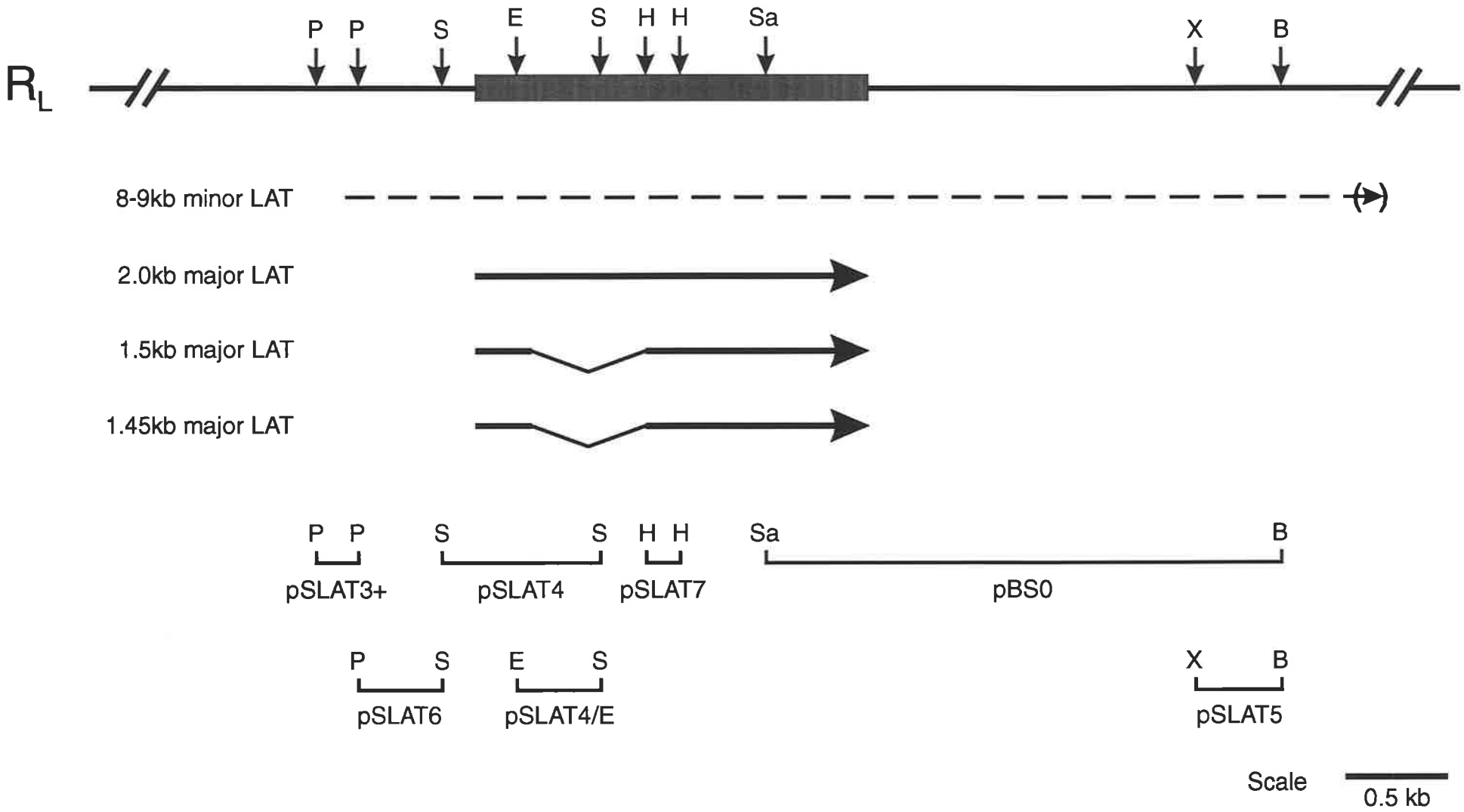
Female BALB/c and Swiss outbred mice were obtained from the Animal Resource Centre, Gilles Plains, South Australia and the Animal Resource Centre, Perth, Western Australia. Mice were bred under specific pathogen-free conditions and the genetic authenticity of the strains was verified by the supplier at six monthly intervals. All mice were used at greater than eight weeks of age.

2.1.4 Plasmids used for production of riboprobes

Cloned HSV-1 restriction fragments used as templates for the production of riboprobes (Figure 2) are as follows (the numbering system of McGeoch *et al* (1988) has been used to define positions of fragments in the internal repeat region of the HSV genome, square brackets):

pBS0 — 2.55 kb SalI-BamHI fragment [120904-123460] from strain KOS BamHI B fragment cloned into pBS (Stratagene, La Jolla, California, USA).

Figure 2. Part of the long repeat region (R_L) of the HSV-1 genome showing the relative positions of the major LAT region (boxed) and the restriction enzyme sites used to construct the six BamHI B subclones (shown below) from which riboprobes were generated (P, S, E, H, Sa, X, B represent Pst I, Sph I, Eco 52I, Hpa I, Sal I, Xho I and Bam HI respectively). In some experiments truncated probes were generated from pSLAT4 and pBS0 templates linearized prior to transcription with Eco 52I (pSLAT4/E) and Xho I (pBS0/X) respectively. Also depicted are the positions of major LATs (solid arrows) and minor LATs (broken line). Minor LATs extend rightwards (→) through R_L beyond the scope of the diagram.


R_L
P P S E S H H Sa X B
8-9kb minor LAT
2.0kb major LAT
1.5kb major LAT
1.45kb major LAT
P P S S H H Sa B
pSLAT3+
pSLAT4
pSLAT7
pBS0
P S E S X B
pSLAT6
pSLAT4/E
pSLAT5
Scale
0.5 kb

pSLAT3^{+} 0.2 kb PstI fragment [118665-118865] from SC16 BamHI B fragment cloned into pGEM4 (Promega, Madison, Wisconsin, USA).

pSLAT4 0.79 kb SphI fragment [119292-120078] from HSV-1 strain 17 BamH1 B fragment cloned into pBS.

pSLAT5 0.43 kb Xhol-BamH1 [123030-123460] fragment from pBS0 cloned into pBS.

pSLAT6 0.42 kb Pst-Sph1 fragment [118868-119292] from HSV-1 strain SC16 BamHI B cloned into pBS.

pSLAT7 0.17 kb Hpa1 fragment [120302-120470] derived from SC16 BamHI B restriction fragment cloned into pBS.

Transcription of pSLAT6 and pSLAT7 templates from the T3 promoter generated probes complementary to LATs; all other inserts were orientated such that T7 transcripts were complementary to LATs. Plasmids were linearized by restriction enzyme digestion at a site 3' to the HSV-1 insert and gel-purified to remove uncut templates prior to transcription. In some experiments truncated probes were produced from pSLAT4 linearized with Eco52I (pSLAT4/E), and pBS0 linearized with XhoI (pBS0/X). pBS0/X and pSLAT5 probes were equivalent.

Plasmids pSLAT3^{+},4,5,7 and pBS0 were gifts from Dr Stacey Efstathiou, Cambridge University, Cambridge, UK.

2.1.5 Sera and antibody conjugates

For detection of digoxigenin

The following polyclonal anti-digoxigenin (anti-DIG) immunoglobulin fragments were

obtained from Boehringer (Mannheim, Germany):

anti-DIG-AP — Sheep anti-digoxigenin alkaline phosphatase conjugated Fab fragments (Catalogue number [Cat. #] 1093274).

anti-DIG-POD — Sheep anti-digoxigenin peroxidase conjugated Fab fragments (Cat. # 1207773).

anti-DIG-FITC — Sheep anti-digoxigenin fluorocein conjugated Fab fragments (Cat. # 1207741).

For HSV antigen detection

The following reagents were obtained from Dakopatts (Glostrup, Denmark)

Rabbit immunoglobulins to herpes simplex virus type 1 (MacIntyre strain) infected cells (Cat. # B114).

Swine immunoglobulins to rabbit immunoglobulins (Cat. # Z196).

Soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (Cat. # Z113).

For detection of nuclear antigens

The following sera were obtained from Immuno Concepts:

No — Human autoimmune serum containing antibodies directed against nucleolar antigens including fibrillarin.

CC — Human autoimmune serum containing antibodies directed against antigens contained in the kinetochore complex of chromosomal centromeres.

Rabbit anti-human immunoglobulins conjugated to FITC (Cat# 2009).

The following human sera were a gift from Grace Chang, Division of Human Immunology, IMVS, Adelaide, SA, Aust.

NHS Pooled normal human sera which does not display anti-nuclear antigen activity.

Lane Autoimmune serum from a patient with systemic lupus erythematosus (SLE) which contains high titres of anti-Sm antibodies (as determined by double diffusion assay, G. Chang personal communication) plus weak anti-DNA activity.

Peroxidase conjugated rabbit anti-human immunoglobulins were obtained from Dako (Cat # P214).

2.1.6 Enzymes

Restriction endonucleases were obtained from Amersham (Little Chalfont, UK)

RQ1DNAse, RNA Polymerases T7 and T3 were obtained from Promega.

DNAse 1 and RNAse A used for nuclease digestion of tissue sections prior to *in situ* hybridization were obtained from Boehringer and Pharmacia (Uppsala, Sweden) respectively.

Trypsin was obtained from Gibco BRL (Gaithersburg, Maryland, USA).

Proteinase K was obtained from Merk (Darmstadt, Germany).

2.1.7 Buffers, fixatives and solutions

Buffers

The following buffers were prepared as according to Sambrook *et al* (1989).

Hybridization buffer: SSC, 100mM Tris-HCl pH 7.6, 10mM NaH_2PO_4, 10mM Na_2HPO_4, 0.02% ficoll, 0.02% polyvinyl pyrrolidone.

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

SSC: 150mM NaCl, 15mM trisodium citrate.

Tris-HCl: 1M Tris(hydroxymethyl)aminomethane (Tris) modified to pH with HCl. 1M Tris-HCl stock solutions were prepared at pH 7.4, pH 7.5, pH 8 and pH 9.5.

TBS (Tris-HCl buffered saline): 50mM Tris-HCl pH 7.4, 150mM NaCl.

TE: 10mM Tris-HCl pH 8, 1mM EDTA pH 8.0.

TMN: 100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$.

Transcription buffer: 40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl.

Fixatives

EAA (ethanol-acetic acid): 3:1 mix of ethanol and glacial acetic acid.

Formal saline: 10% formalin in PBS.

4%PB (4% paraformaldehyde, buffered): 4% paraformaldehyde, 100mM KCl, 20mM 1,4-piperazinediethanesulphuric acid (PIPES) pH 7.4, 15mM $MgCl_2$, 0.5mM ethylenebis (oxyethylenenitrilo) tetra-acetic acid (EGTA), 15mM β-mercaptoethanol (Matera and Ward, 1993).

PLP (periodate-lysine-paraformaldehyde): 10mM $NaIO_4$, 75mM lysine, 1% paraformaldehyde, 37mM phosphate buffer, pH 7.4 (McLean and Nakane, 1974)

Solutions

BCIP: 50 mg/ml 5-bromo-4-chloro-3-indoyl-phosphate in dimethylformamide.

Blocking reagent: 1% blocking reagent (Boehringer. Cat# 1096 176) in TBS.

DIG-11-UTP: 10mM digoxigenin-3-O-methylcarbonyl-ε-amino-caproyl-[5-(3-aminoallyl)-uridine-5'-triphosphate] (Boehringer. Cat# 1209 256).

HEPES buffered DMEM: 20mM [N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid] (HEPES)-buffered Dulbecco modified Eagle medium (DMEM, CYTOSYSTEMS, Castle Hill, NSW, Aust. Cat# 50-013-PB) with 10mM $NaHCO_3$, 12 μg/ml penicillin, 16 μg/ml gentamicin, and adjusted to pH 7.2 with 10M NaOH.

Hybridization mix: 50% deionized formamide (Davis *et al*, 1986), SSC, hybridization buffer, 0.5 mg/ml sheared denatured salmon sperm DNA, 0.5 mg/ml *E.coli* tRNA, 20mM DTT, 1 unit/μl RNAsin, 1-30 pg DIG-labelled RNA probe.

Mayer's Haematoxylin: 5% aluminium ammonium sulphate, 0.1% haematoxylin, 0.02% sodium iodate, 1% citric acid, 5% chloral hydrate in DDW (Bancroft, 1990).

NBT: 77 mg/ml nitroblue tetrazolium chloride dissolved in 70% dimethylformamide in DDW.

Toluidine Blue: 1% toluidine blue, 1% $Na_2B_4O_7$ in DDW.

The following solutions were obtained from Promega:

10mM rATP; 10mM rCTP; 10mM rGTP; 10mM rUTP; RNAse-free H_2O; RNAsin (Ribonuclease inhibitor, 40 units/μl).

2.2 METHODS

2.2.1 Preparation of glass microscope slides and coverslips

Glass microscope slides and coverslips were washed in dichromic acid (350mM potassium dichromate, 10% sulphuric acid) overnight, washed in four changes of DDW and air dried. Slides were dipped in a 2% solution of 3-aminopropyltriethoxysilane (APES) in ethanol for 10 sec, rinsed in ethanol then DDW and allowed to dry.

APES-coated slides were activated by immersion in 10% solution of glutaraldehyde (in PBS) for 30 min, not more than 24 hr before use (Maples, 1985).

Dichromic acid washed coverslips were siliconized by dipping in 1% Prosil^{R} (PCR Inc., Gainesville, Florida, USA), rinsed in DDW, dried at 80°C for 1 hr and dry heat sterilized at 160°C for 2 hr.

2.2.2 Cell culture

Vero and Neuro-2a lines

Continuous cell lines (Vero and Neuro-2a) were routinely grown in 150 cm^2 tissue culture flasks (Corning) with 50 ml of HEPES buffered DMEM supplemented with 10% foetal calf serum (FCS, CSL, Parkville, Vic., Aust.) in a 37°C humidified incubator with 5% CO_2. Culture flasks were seeded with 2.5 x 10^6 Vero cells or 4 x 10^6 Neuro-2a cells and subcultured when monolayers reached confluency (after 3 to 4 days). Confluent monolayers were washed twice with PBS (1 min) then dissociated with 0.1% trypsin (in PBS with 0.5mM EDTA, for 1 to 5 min) and the harvested cells resuspended in HEPES

buffered DMEM containing 10% FCS prior to subculture.

Preparation of Neuro-2a monolayers for immunohistochemistry

Glass microscope slides were placed into 20 cm diameter glass petri dishes and dry heat sterilized at 160°C for 2 hr. Dishes were seeded with 8 x 10^6 Neuro-2a cells in 100 ml HEPES buffered DMEM supplemented with 10% FCS and incubated as described above until cell monolayers on slides reached confluency (after 3 to 4 days). Monolayers were rinsed in PBS (1 min) prior to use.

2.2.3 Quantification of infectious virus

Infectious virus was quantified by duplicate plaque assay using the suspension method of Russell (1962). Virus suspensions were serially diluted (tenfold) in HEPES buffered DMEM containing 1% FCS. $3x10^6$ Vero cells were added to each dilution, in a total volume of 1ml, and the samples shaken vigorously for 1 hr at room temperature. 4mls of HEPES buffered DMEM containing 10% FCS, 1% carboxmethylcellulose (medium viscosity) was added and the full 5ml plated into plastic Petri dishes (60 mm diameter, Nunc, Roskilde, Denmark) and incubated at 37°C for 2.5 days in a humidified atmosphere containing 5% CO_2. Cell monolayers were fixed with 10% formal saline for 30 min, rinsed in tap water and stained in 0.1% toluidine blue for 30 min. Plaques were counted using a plate dissecting microscope.

2.2.4 Infection of mice

Mice were infected using the zosteriform model of HSV infection as described previously (Simmons and Nash, 1984, 1985; Simmons, 1985; Simmons and La Vista, 1989; Scalzo *et*

al, 1990). Briefly, after depilation of the left flank with Nair (Carter-Wallace, Frenchs Farm, NSW, Aust.) the mice were lightly anaesthetised with ether vapour. A small area of skin over the spleen tip, within the left tenth thoracic dermatome, was scarified (20 times) with a 27-gauge needle through a 10 μl drop of virus suspension containing 3.5 x 10^4 to 1.5 x10^5 pfu. Five to six days following inoculation infected mice develop a band-like zosteriform lesion around the left flank corresponding to the dermatome inoculated. By eight days after infection viral antigens are no longer detectable in ganglia (Speck and Simmons, 1992).

2.2.5 Removal of ganglionic tissues

Mice were humanely killed by CO_2 asphyxiation or by intraperitoneal (ip) injection of 200 μl of sodium pentibarbitone (60 mg/ml). The viscera were removed through an anterior midline incision to expose the anterior surface of the spine. The thirteenth thoracic vertebra (T13) was identified by its articulation with the 13th (lowest) rib. Fine forceps were used to gently separate the vertebral discs and remove dorsal root ganglia (drg).

2.2.6 Preparation of paraffin embedded tissues

PLP fixed murine tissues

At various times after inoculation pooled, ipsilateral dorsal root ganglia (drg) from the sixth thoracic (T6) to first lumbar (L1) regions were collected into freshly prepared PLP and incubated for 60-120 min at room temperature (McLean and Nakane, 1974). Drg tissues from uninfected BALB/c mice were taken from both sides between T6 and L1 and similarly fixed. PLP was chosen for its suitability for preservation of nucleic acids and antigens (McAllister and Rock, 1985; Moench *et al*, 1985; Putchler and Meloan, 1985;

Raymond and Leong, 1988). Fixed ganglia were rolled into a ball and stored (up to 24 hr) in 50% ethanol until embedded in paraffin.

EAA fixed murine tissues

Murine drg were removed collected into freshly prepared EAA and fixed for 1 hr at room temperature (Gowans *et al*, 1989). EAA-fixed tissues were stored in 50% ethanol until processed into paraffin.

PLP fixed human tissues

Trigeminal ganglia, removed from two HSV-seropositive cadavers during postmortem examination of the nervous system (within 48 hr after death) were fixed in PLP overnight (about 16 hr) at room temperature, transferred to 50% ethanol until processed into paraffin. The cause of death was determined to be (i) pulmonary embolus and (ii) pneumonia. A blood sample from each cadaver, taken at autopsy, was assayed for the presence of anti-HSV immunoglobulins by staff of the Serology Laboratory, Division of Medical Virology, IMVS, Adelaide, SA. Both sera contained immunoglobulins to HSV antigens. Ten randomly chosen sections from each tissue were examined immunohistochemically for the presence of HSV antigens, all were negative (see 2.2.8 for method).

Fixed 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.

Sections (5 μm) of tissue were collected onto glutaraldehyde-activated APES coated microscope slides (see section 2.2.1).

2.2.7 Preparation of frozen tissues

At various times after infection pooled, ipsilateral drg were collected into cold PBS (on ice), gathered into a ball and embedded in optimum cutting temperature (OCT) compound (Miles Inc, Iowa, USA). Ganglia were rapidly frozen in liquid nitrogen-cooled isopentane. This method of freezing was chosen for maximum preservation of tissue structure (Menco, 1986). Frozen blocks were stored temporarily at -70°C and used within five days of collection.

Sections (6 μm) were cut at -20°C (Ames cryostat II, Miles Inc.) and affixed to air-dried, glutaraldehyde-activated APES-coated slides.

2.2.8 Immunohistochemical detection of viral antigens

All tissues were assessed for the presence of viral antigens using the peroxidase anti-peroxidase detection system (Moriarty *et al*, 1973; Sternberger, 1979; Boenisch, 1980). Dewaxed, rehydrated tissue sections were blocked for endogenous peroxidase activity (0.1% H_2O_2 in TBS, 15 mins) and spurious binding of antibodies (20% pooled normal swine sera in TBS, 30 min at 37°C) prior to application of rabbit immunoglobulins to HSV-infected cells. Binding of primary antiserum was detected using swine anti-rabbit serum followed by peroxidase-anti-peroxidase (rabbit) complex. Antisera were diluted in 20% pooled normal swine serum (in 50mM Tris-HCl pH 7.5). Reactions were allowed to proceed for 30 min at 37°C, with two 5 min washes in 50mM Tris-HCl (pH 7.5) between each incubation. Bound antibody was detected with 3,3'-diaminobenzidine (0.5 mg/ml in 50mM Tris-HCl pH 7.5, containing 0.1% H_2O_2) and sections were counterstained in Mayer's haematoxylin (30 sec).

2.2.9 *In situ* hybridization to PLP-fixed, paraffin-embedded tissues

Choice of molecular probe

For the detection of LATs, strand-specific anti-sense riboprobes were used throughout. Riboprobes of the same sense as LATs were used to control for spurious hybridization (Gowans *et al*, 1989). It has been shown that, being single-stranded, riboprobes have less tendency to self-anneal than denatured double stranded DNA probes, which effectively increases probe concentration, thereby maximizing sensitivity (Angerer *et al*, 1987).

Choice of indicator molecule

Non-isotopic digoxigenin (DIG) was chosen as the indicator molecule for *in situ* hybridization. DIG-11-UTP is readily incorporated into RNA during transcription and following *in situ* hybridization (ISH) bound riboprobes were detected with FITC- or enzyme-conjugated anti-DIG Fab fragments, which provide good localization of the hybridization signal. The rapid detection of bound probe in the DIG system (~3 to 5 hr) was considered an advantage compared with the prolonged exposure times (up to several weeks) required for isotopic labels capable of high resolution (eg., ^{3}H). Further, DIG-labelled riboprobes have a half-life of up to one year which provides continuity between experiments because sequential ISH reactions can be done with the same batch of probe.

Preparation of riboprobes

Linearized, gel purified templates were used for the transcription of riboprobes. The transcription reaction contained:

4 μl 5x transcription buffer; 1 μl 25 units/μl RNAsin; 1 μg of linearized template; 1 μl (20 units) T7 or T3 RNA polymerase; 2.5mM each of rATP, rCTP and rGTP; 12μM rUTP; 250μM DIG-11-UTP; 0.085μM-[^{32}P]rUTP (5 μCuries; Bresatec, Adelaide, SA, Aust.);

RNAse-free H_2O to 20 μl.

Transcription was done at 37°C for 1 hr. 1 μl (1 unit) of RQ1 RNA-free DNAse was then added to digest the template (15 min, 37°C). The reaction was stopped by the addition of 1 μl of 500mM EDTA pH 9.5 and RNA was precipitated overnight at -20°C with 400mM LiCl and 3 volumes of ethanol. RNA was pelleted by centrifugation at ~13,000$_G$ for 20 min. The pellet was washed twice in 70% ethanol to remove unincorporated DIG-11-UTP, dried and resuspended in 50 μl RNAse-free H_2O plus 0.1μM DTT and 40 units of RNAsin. Probe stocks were stored at -70°C for up to 12 months.

Activity of DIG-labelled riboprobes

To ensure that riboprobes were labelled trace amounts of ^{32}P-rUTP (was added to the transcription reaction. In the reaction described above, the ratio of cold UTP (ie., rUTP plus DIG-11-UTP) to ^{32}P-rUTP was approximately 3000:1. The level of ^{32}P-rUTP alone is sufficient for the synthesis of ~2 ng of RNA. From each transcription reaction 0.2 to 2.0 μg of RNA was recovered, as determined by the incorporation of the radioactive tracer (assuming that [^{32}P]rUTP and DIG-UTP were equally incorporated).

In situ hybridization

Sections were dewaxed in xylene and rehydrated in graded alcohol solutions, refixed in 0.1% glutaraldehyde (in PBS) for 30 min at 4°C and washed in PBS (5 min). Tissue was digested with 100 μg/ml proteinase K (15 min, 37°C, in 20mM Tris-HCl pH 7.5, 2mM $CaCl_2$), washed in PBS (1 min), refixed in 0.1% glutaraldehyde (in PBS, 15 min at 4°C), and rinsed in 0.1xSSC before acetylation (10 min in 0.25% acetic anhydride, 100mM triethanolamine, pH 8.0). After acetylation, slides were washed in SSC (10 min) and drained thoroughly before the addition of 20 μl hybridization mixture containing 1 to 50 ng

of DIG-labelled RNA probe per section. Lower concentrations of probe (less than 1 ng per section) were associated with reduction of detectable hybridization and extended development times (up to 24 hr, not illustrated). Background staining in ISH increases linearly as a function of probe concentration (Cox *et al*, 1984) and hybridization with probe concentrations of 100 ng per section and above were associated with an increase in background staining over uninfected neurons and nerve fibres (not illustrated). The concentration of salt in the hybridization buffer was selected to give hybridization temperatures compatible with good tissue preservation.

50x22mm glass coverslips coated with Prosil (PCR Inc.) were sealed in place with rubber cement (Vulkarn, Maruni Industries, Osaka, Japan) and sections were incubated for 16 hr at 22 to 26°C below the theoretical Tm_{50} (see *Calculation of* Tm_{50} below) ie., pBS0, 74°C; pSLAT3^{+}, 60°C; pSLAT4 and 4/E, 67°C; pBS0/X, pSLAT5 and pSLAT6, 71°C; pSLAT7, 69°C.

Washing

Following hybridization unbound probe was removed by washing slides sequentially at room temperature in SSC (5 min), 0.1xSSC containing 10mM Tris-HCl (pH 7.5, 60 min), followed by a stringent wash (30 min) in 0.1xSSC, 30% deionized formamide, 10mM Tris-HCl pH 7.5, at 5 to 15°C below Tm_{50} (see *Calculation of* Tm_{50} below) ie., pBS0, 74°C; pSLAT3^{+}, 60°C; pSLAT4, pSLAT4/E and pSLAT7, 68°C; pBS0/X, pSLAT5 and pSLAT6, 70°C.

Detection of hybridized probe

Bound probe was detected with anti-DIG Fab fragments coupled either to alkaline phosphatase or peroxidase. Stringently washed sections were rinsed in 0.1xSSC containing

10mM Tris-HCl pH 7.5 (15 min), blocked for non-specific binding of protein (with blocking reagent) for 30 min at room temperature. Anti-DIG Fab fragments were diluted in blocking reagent and incubated with sections for 30 min at 37°C. Slides were washed thoroughly in TBS (30 min). For alkaline phosphatase labelled antibody, slides were transferred to TMN pH 9.5 containing 0.17 mg/ml BCIP and 0.35 mg/ml NBT (as recommended by the manufacturer Boehringer) and developed under lightproof conditions. For peroxidase labelled antibody, slides were rinsed in 50mM Tris-HCl pH 7.5 (1 min) then incubated in 50mM Tris-HCl pH 7.5 containing 0.5mg/ml diaminobenzadine (DAB) and 0.1% H_2O_2 (10 min). Slides were washed thoroughly in tap water to stop development and if required, sections were counterstained with Mayer's haematoxylin (10 sec).

Nuclease digestions

Where indicated, tissues were treated for 45 min at 37°C with 200 μg/ml RNAse A in 2xSSC or, 100 μg/ml DNAse 1 in 40mM Tris-HCl pH 7.6, 10mM NaCl, 6mM $MgCl_2$, prior to proteinase K digestion.

Calculation of Tm_{50}

Tm_{50} is the temperature at which half of double-stranded RNA hybrids, in liquid, will dissociate into single-stranded molecules. The theoretical Tm_{50} is defined by the equation:

$$Tm_{50}\ (\text{RNA-RNA}) = 79.8 + 18.5\log[Na^+] - 0.35(\%\ \text{deionized formamide}) + 0.584(\%G+C) + 0.0012(\%G+C)^2$$

(Bodkin and Knudson, 1985), where [Na+] is the concentration of sodium ions in the solution and %G+C is the percentage of guanidine plus cytosine residues in the nucleic acid sequence. The optimum temperature for hybridization has been determined to be about 20 to 25°C below the Tm_{50} (Britton and Davidson, 1986).

2.2.10 *In situ* hybridization detection of viral RNAs in frozen tissues

Sections (6 μm) of frozen drg were air dried (10 min) then fixed in 4%PB (15 min), washed in 100mM glycine (5 min), rinsed in PBS (1 min). Fixed sections were acetylated (10 min in 0.25% acetic anhydride, 100mM triethanolamine, pH 8.0), rinsed in PBS (5 min) and thoroughly drained prior to the application of 1 to 50 ng of probe in 20 μl hybridization mix. Sections were covered with 50x22mm Prosil-coated glass coverslips, sealed in place with rubber cement (Vulkarn) and the slides were incubated overnight (about 16 hr) at 22 to 26°C below Tm_{50}.

Following hybridization, coverslips were gently removed and sections were rinsed in PBS (15 min). Sections were stringently washed (as per section 2.2.9: *Washing*), rinsed thoroughly in PBS (10 min) and bound probe detected with anti-DIG-AP as described (section 2.2.9: *Detection of hybridized probe*) or incubated with anti-DIG-FITC (diluted in blocking reagent) for 30 min, rinsed in PBS and mounted in 90% glycerol (in PBS, pH 9).

FITC excitation was viewed using 490nm incident illumination and a 515nm barrier filter.

2.2.11 Immunohistochemical detection of nuclear antigens in cells in culture and frozen tissues

Neuro-2a cell monolayers and sections (6 μm) of frozen murine drg were fixed in acetone (10 min, 4°C) and air dried. Human autoimmune sera were applied (in PBS, 37°C for 15 min) and binding of primary antiserum was detected using peroxidase-conjugated or FITC-conjugated rabbit anti-human antisera. Secondary antibodies were applied for 30 min at 37°C with two 5 min washes in TBS (pH 7.5) between steps. Bound peroxidase-conjugated

antibody was detected with 3,3'-diaminobenzidine (0.5mg/ml in 50mM Tris-HCl pH 7.5, containing 0.1% H_2O_2) and sections were counterstained with Mayer's haematoxylin (30 sec). Sections treated with FITC-conjugated antibody were mounted in 90% glycerol (in PBS, pH 9) and bound antibody visualized directly by fluorescence microscopy.

2.2.12 Photography

Photographic films (KODAK, Rochester, New York, USA) techpan ASA 100 (black and white), Ektachrome ASA 64 (colour slide film) and EPL ASA 400 (fluorescence) were used for the preparation of photomicrographs. Photographic films were developed and printed by staff of the IMVS Photographic Unit (Adelaide, South Australia). Colour prints were generated from colour transparencies (slides) and digital laser copied by Colour Quick (Adelaide, South Australia) for presentation in this thesis.

3 ANALYSIS OF THE INTRACELLULAR DISTRIBUTION OF LATS

Previous *in situ* hybridization studies (ISH) using radioactively labelled probes complementary to LATs indicated that LATs may be confined to the nuclei of latently infected neurons. In order to examine the subcellular distribution of LATs in detail, a method using high resolution non-isotopic ISH was developed. Using this procedure the intracellular distributions of RNAs arising from three regions within the LAT locus were examined:

(i) The major LAT region.

(ii) Upstream minor LAT sequences between the LAT promoter and the 5' end of the major LATs.

(iii) Sequences from ~1.6 to 2 kb downstream of the 3' end of the major LATs.

3.1 DETECTION OF LATS BY NON-ISOTOPIC *in situ* HYBRIDIZATION

The intracellular location of LATs was analysed in PLP fixed latently infected dorsal root ganglia using high resolution non-isotopic ISH. Inbred (BALB/c) and outbred (Swiss) mice were studied at times ranging from 44-660 days after infection as follows:

(i) A group of eleven BALB/c mice 44 days after infection.

(ii) A group of eight BALB/c mice 207 days after infection.

(iii) A group of eight Swiss outbred mice 660 days after infection.

Five randomly chosen sections from each block were assessed immunohistochemically for the presence of HSV antigens, all were negative.

DIG labelled RNA probes complementary to major and minor LAT sequences generated from pSLAT4, stained neuronal nuclei (Figure 3.1 a&c) diffusely. Based on the distribution of staining detected by pSLAT4 probes it was proposed that major and/or minor LATs are distributed throughout neuronal nucleoplasms. This conclusion was strengthened by analysis of tissues using probes generated from templates pSLAT4/E and pSLAT7 (Figures 3.2 and 3.3) with each probe (pSLAT4, 4/E, 7), staining in the nucleoplasm was diffuse and punctate, often with prominent sparing of nucleoli (Figure 3.2 a&b). The hybridization signal was predominantly nuclear and a wide variation in the intensity of staining among LAT^+ neurons in each ganglionic section was observed (Figure 3.2c). Weak staining was sometimes uneven or patchy (Figures 3.2c and 3.10b). In neurons displaying intense hybridization signal, staining often extended slightly beyond the nuclear membrane into the cytoplasm (eg., Figures 3.2b and 3.3). Overall, the staining pattern was consistent in all tissues examined, indicating that the distribution of LATs was stable for extended periods irrespective of the mouse strain.

RNA probes anti-sense to major LATs (generated from pSLAT4) did not hybridize to ganglionic tissues taken from four uninfected BALB/c mice (Figure 3.1b), indicating that hybridization signal was an exclusive feature of HSV-infected tissue. Sections from uninfected animals were included in all *in situ* hybridization experiments as a control for spurious hybridization (background).

The specificity of hybridization for RNA was assessed in two ways. First, RNA probes of the same sense as LATs (generated from pSLAT4) were applied to sections of latently

Figure 3.1. Light micrographs illustrating detection of LATs by ISH in sections (5 μm thick of murine dorsal root ganglia (drg).

(a) Drg removed from BALB/c mice 44 days after infection showing latently infected primary sensory neurons (arrowhead) in which the nucleus is densely stained (black) detected with a probe antisense to LATs generated from pSLAT4.

(b) A ganglionic profile from an uninfected BALB/c mouse showing no hybridization (arrowhead) when probed with a LAT-specific probe generated from pSLAT4.

(c) A ganglionic profile from a Swiss outbred mouse 660 days after infection showing intense staining (black) over the nucleus of a neuron (arrowhead) when probed with a LAT-specific probe generated from pSLAT4.

(d) A ganglionic profile from a Swiss outbred mouse 660 days after infection showing no hybridization when treated with RNAse prior to hybridization with a LAT-specific probe generated from pSLAT4.

Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments. Magnification is (a and e) 70x; (b) 200x; (c) 400x.

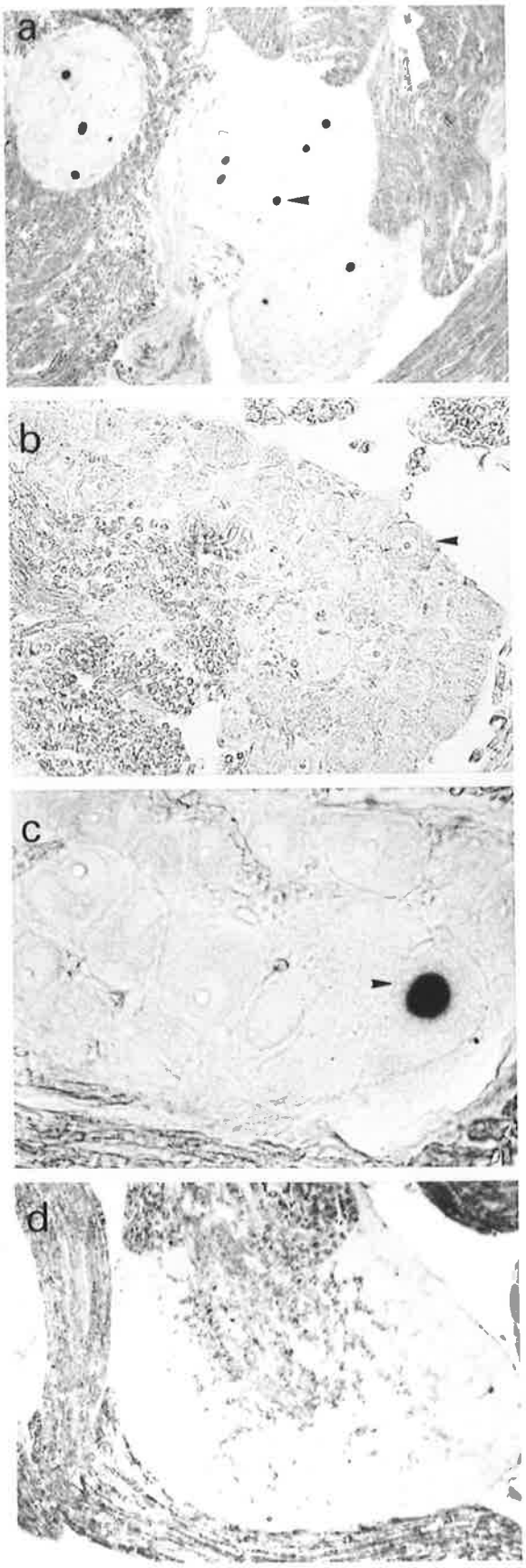

a
b
c
d

Figure 3.2. Light micrographs illustrating detection of LATs by ISH.

(a) Section of a spinal ganglia removed from a BALB/c mouse 44 days after infection showing a latently infected primary sensory neuron (arrowhead) in which the nucleoplasm is diffusely stained (arrow) by a probe antisense to LATs generated from pSLAT7.

(b) Section of a spinal ganglia removed from a Swiss outbred mouse 660 days after infection, showing diffuse nucleoplasmic staining of two neurons by a probe antisense to LATs generated from pSLAT4/E. Staining typically spared nucleolar regions (arrow).

(c) BALB/c ganglionic section, 44 days after infection stained with a probe antisense to LATs generated from pSLAT7, showing three neurons (boxed) that typify the range in the intensity of LAT staining.

Bound probe was detected using alkaline phosphatase-conjugated anti-DIG Fab fragments. Magnification is (a and b) 1000x; (c) 250x.

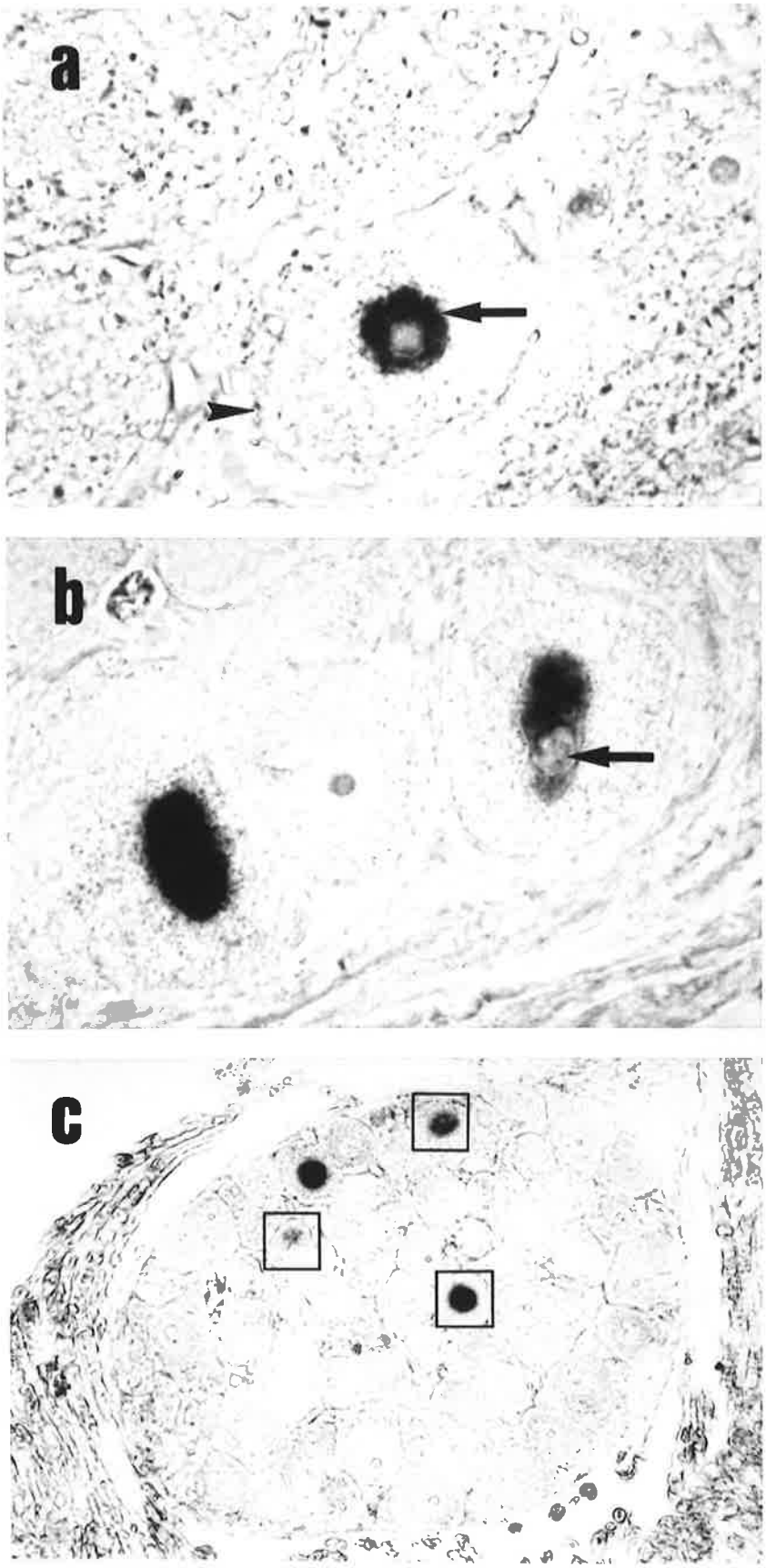
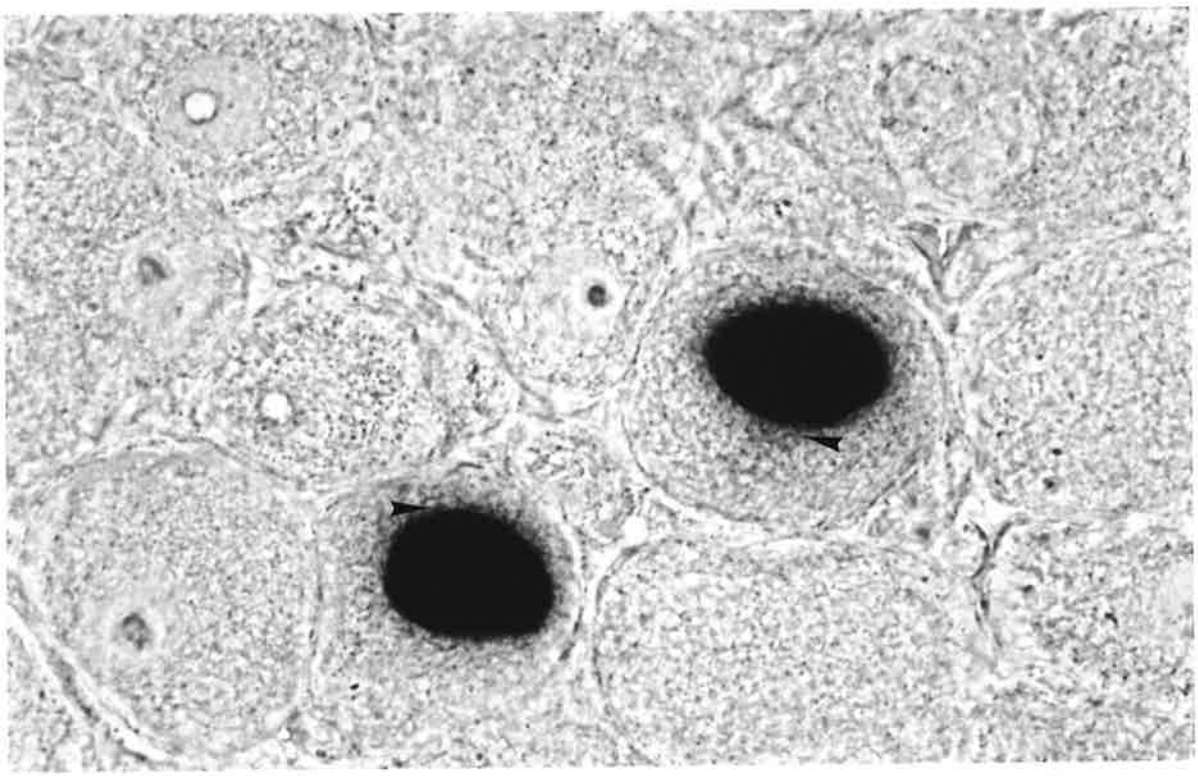

a
b
c

Figure 3.3. Light micrograph illustrating detection of LATs by ISH in a ganglionic profile (5 μm thick) from a BALB/c mouse 207 days after infection. Neuronal profiles displaying intense staining of nucleus (black) plus weak staining of the cytoplasm surrounding the nucleus (arrowheads) produced by hybridization with a LAT-specific probe generated from pSLAT4/E. Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments. Magnification is 1200x.



infected ganglionic tissue (taken from eleven BALB/c mice 44 days after infection) (Figure 3.1d). No hybridization was detected, indicating that the molecules detected by probes complementary to the LAT region were not double stranded DNA. Second, digestion of sections of latently infected ganglia with ribonuclease A (RNAse A; see section 2.2.9 *Nuclease digestion*) prior to the application of probe completely abolished the hybridization signal (not illustrated). This loss was not a result of degradation of the RNA probe by residual RNAses in the tissue, because when the probe was applied to similarly treated sections of acutely infected (HSV antigen positive) ganglionic tissue (taken from 8 BALB/c mice 5 days after infection) hybridization to HSV DNA sequences was preserved (not illustrated). Further, predigestion of sections of latently infected tissue with deoxyribonuclease I (DNAse I; see section 2.2.9 *Nuclease digestion*) did not appreciably decrease the intensity of the hybridization signal (not illustrated).

From the preceding results, it was concluded that (i) RNA probes anti-sense to major LAT sequences specifically hybridize to HSV infected tissues, (ii) the target nucleic acids in latently infected tissues are RNA and not DNA molecules and therefore (iii), the staining associated with pSLAT4, pSLAT4/E and pSLAT7 probes indicates the presence of HSV LATs, distributed diffusely, in the nuclei of latently infected neurons.

3.2 INTRACELLULAR LOCATION OF MINOR LATS

Minor LATs have been detected by ISH in the nuclei of neurons during latent infection. Little is known about the physical nature of these transcripts and their relation to the major LATs is not clear. It has been proposed that minor LATs are nascent RNA molecules from which the major LATs are spliced. It was reasoned that determination of the subcellular location of minor LATs may provide insight into their function.

3.2.1 Detection of minor LAT sequences

Probes complementary to minor LATs arising from sequences between the LAT promoter (LAP1) and the 5' end of major LATs (generated from templates pSLAT3$^+$ and pSLAT6, Figure 3.4a, b&c) and from ~1.6 to 2 kb beyond the 3' end of the major LATs (generated from pSLAT5 templates, Figure 3.4d) were used to analyse the subcellular distribution of minor LAT sequences in the three latently infected tissues previously described (section 3.1).

All probes gave rise to a focal staining pattern, disclosing discrete intranuclear domains of 1 to 3 μm in diameter (Figure 3.4). The average diameter of LAT$^+$ neuronal nuclei was about 20 μm. Foci were not observed in contact with the nuclear membrane.

Further, examination of HSV-1 acutely infected dorsal root ganglia removed from 8 BALB/c mice collected 5 days after infection, using probes generated from pSLAT5 demonstrated a focal staining pattern in a small proportion of neurons in acutely infected ganglia (Figure 3.5).

The specificity of hybridization for minor LATs was confirmed as follows:

(i) Staining was not detected in uninfected tissues (Figure 3.4f), or in latently infected tissues (described previously in section 3.1) incubated with probes of the same sense as minor LATs (not illustrated).

(ii) Digestion of latently infected ganglionic sections (taken from eight Swiss outbred mice 660 days after infection) with DNAse prior to the application of probe did not appreciably decrease the intensity of the hybridization signal (Figure 3.4e).

Figure 3.4. Light micrographs illustrating detection of minor LATs by ISH in ganglia of BALB/c mice 44 days after infection (a to d) and Swiss outbred mice 660 days after infection (e).

(a) Latently infected neurons (arrow) showing sharply defined foci of hybridization produced by a minor LAT-specific probe generated from pSLAT6.

(b) Higher magnification showing minor LAT-specific foci of hybridization (arrow) within a neuronal nucleus (arrowhead) detected with a probe generated from pSLAT6.

(c) A latently infected neuron showing an intranuclear domain hybridized with a minor LAT-specific probe generated from pSLAT3^{+}.

(d) Adjacent latently infected neurons in a ganglionic section showing foci of hybridization produced by a minor LAT-specific probe (generated from pSLAT5).

(e) Latently infected neuron showing foci of hybridization produced by a minor LAT-specific probe (generated from pSLAT5) in a ganglionic section treated with DNAse prior to hybridization.

(f) A ganglionic profile from an uninfected BALB/c mouse illustrating a lack of foci of hybridization when probed with minor LAT-specific probes generated from pSLAT6.

Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments. Magnification is (a) 250x; (b to e) 750x; (f) 160x.

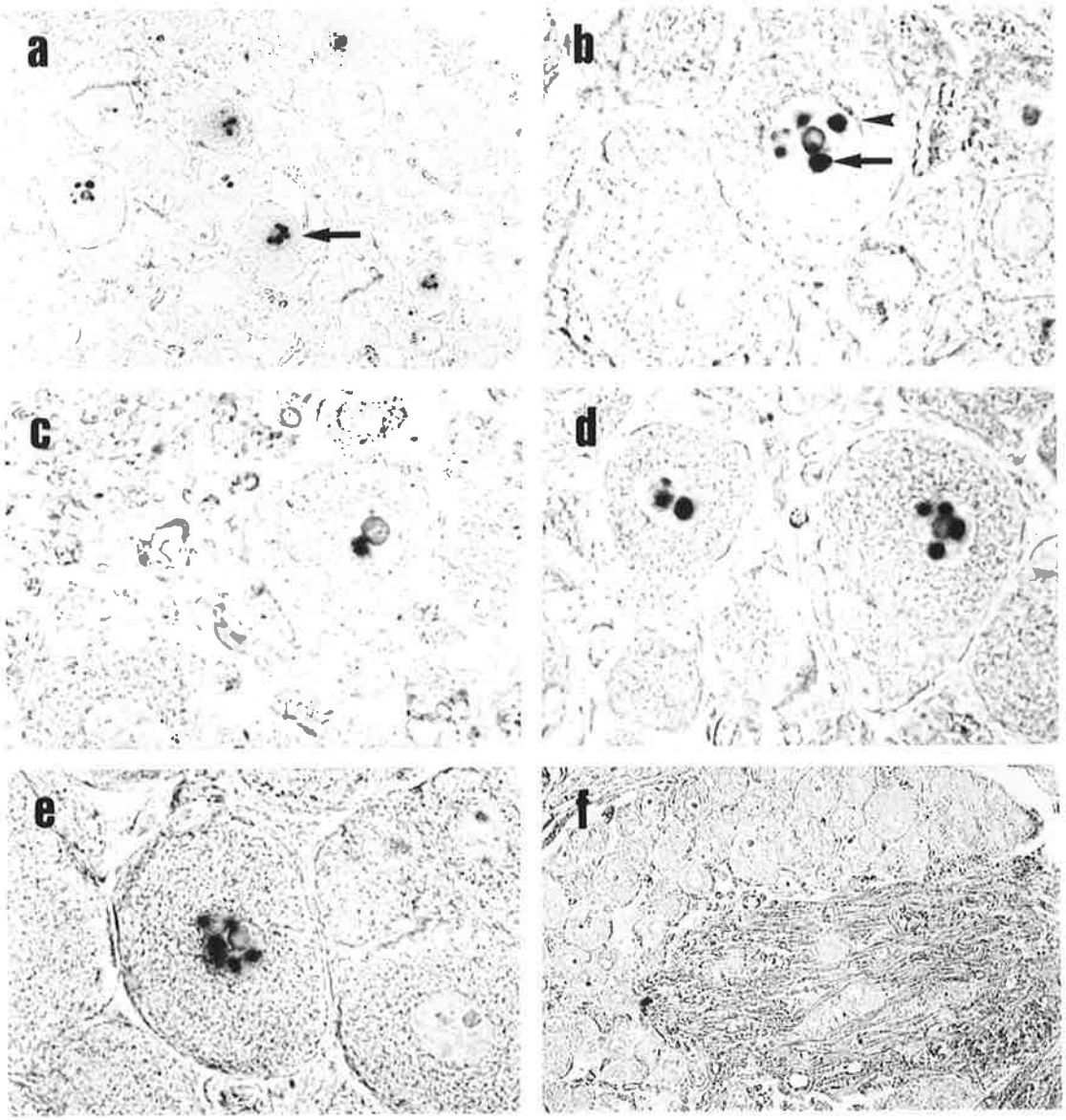
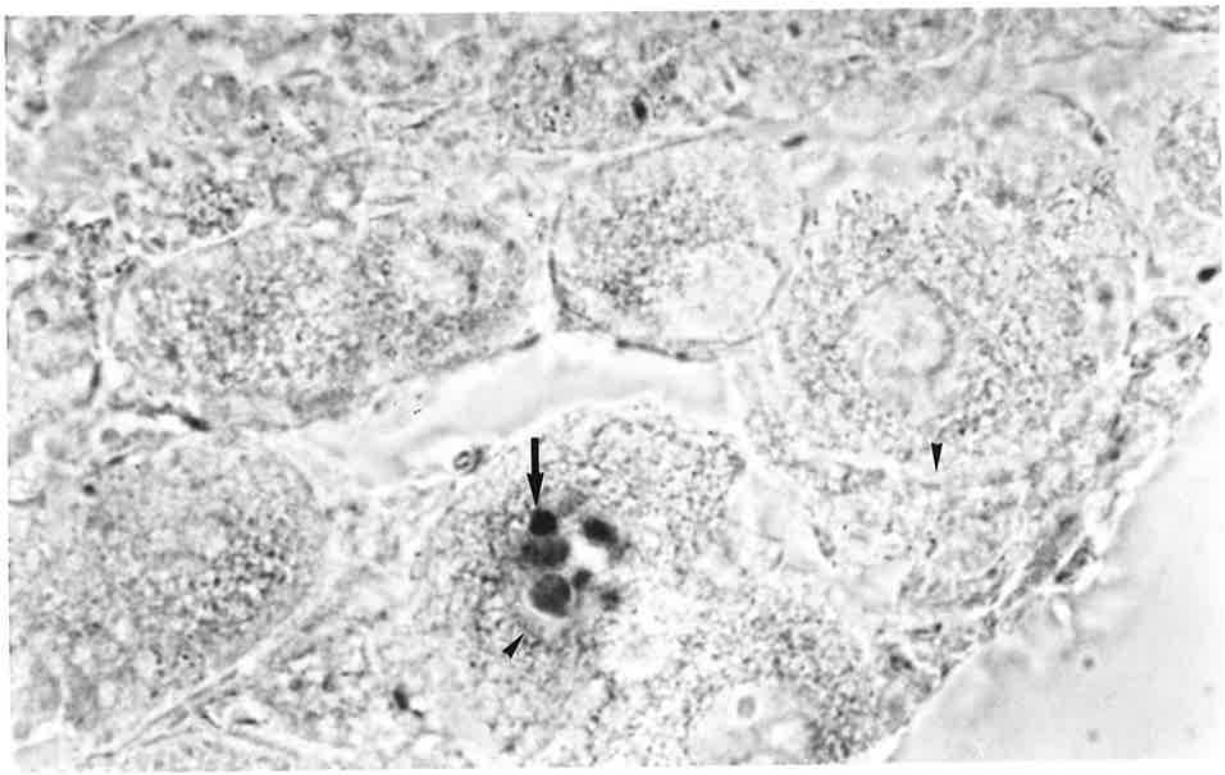

a
b
c
d
e
f

Figure 3.5. Light micrograph illustrating detection of minor LATs in a ganglionic profile (5 μm thick) from a BALB/c mouse 5 days after flank skin inoculation. Foci of hybridization (arrow) within a neuronal nucleus (arrowhead) detected with a minor LAT-specific probe generated from pSLAT5. Magnification is 1200x.



However, pretreatment of latently infected ganglionic sections with RNAse A completely abolished the hybridization signal (not illustrated).

Thus it was concluded that (i) RNA probes anti-sense to minor LAT sequences specifically hybridize to HSV infected tissues, (ii) the target nucleic acids in latently infected tissues are RNA and not DNA molecules, (iii) the minor LATs detected are localized to foci within neuronal nuclei in latently infected ganglionic tissues and, (iv) the focal subnuclear distribution of minor LATs is present in a small proportion of neurons in acutely infected tissues 5 days after inoculation of flank skin.

3.2.2 Effect of tissue fixation on the detection of foci of minor LAT RNA

In all experiments described above, tissues were preserved with PLP, which contains a cross-linking fixative (paraformaldehyde) known to preserve intranuclear RNA-containing domains accurately (Carmo-Fonseca *et al*, 1991b). The validity of PLP fixation was tested by comparing PLP fixed tissues with tissues preserved with a non-crosslinking fixative (EAA) and with sections of frozen ganglia fixed after sectioning with 4% PB. The following tissues and probes were used:

(i) Spinal ganglia removed from 6 BALB/c mice 122 days after infection preserved in ethanol-acetic acid (3:1; EAA), hybridized with pSLAT5 probes.

(ii) Spinal ganglia removed from 8 BALB/c mice 40 days after infection which were frozen, cut and post-fixed with 4%PB, and hybridized with pSLAT6 probes.

(iii) Spinal ganglia removed from 8 BALB/c mice 207 days after infection, fixed with PLP, and hybridized with pSLAT5 probes.

Focal staining for minor LATs (detected using pSLAT5 probes) was observed in neuronal nuclei in EAA fixed tissues (not illustrated). However, in comparison to PLP-fixed tissues, staining was much weaker in EAA-preserved tissues and consequently PLP was regarded as a superior fixative for studying RNA localization by ISH.

Using pSLAT6 probes, focal staining was observed in profiles of neuronal nuclei in 4%PB fixed frozen ganglionic sections. Similar results were obtained irrespective of whether bound probe was detected with alkaline phosphatase- or FITC-conjugated anti-DIG antibodies (Figure 3.6). Preservation of tissue sections following ISH, as assessed histologically, was better in PLP-fixed, wax embedded sections than 4% PB frozen sections. However, frozen sections were considered obligatory for the anti-DIG FITC-conjugate detection system (Figure 3.6c) because autofluorescence of PLP-fixed wax embedded sections was problematic.

These observations indicate that the detection and characteristics of domains containing minor LATs is not influenced by the tissue fixation procedure.

3.2.3 Number and size of foci detected by two different minor LAT probes

Given that, minor LAT sequences upstream (detected with pSLAT3^{+} and pSLAT6 probes) and downstream (detected with pSLAT5 probes) of the major LAT region are localized to intranuclear foci, the staining patterns generated were analysed to determine if upstream and downstream sequences were located within the same nuclear domains.

The staining patterns associated with pSLAT5 and pSLAT6 probes were compared in a concurrent analysis of 183 randomly selected sections taken from ganglia of 11 BALB/c

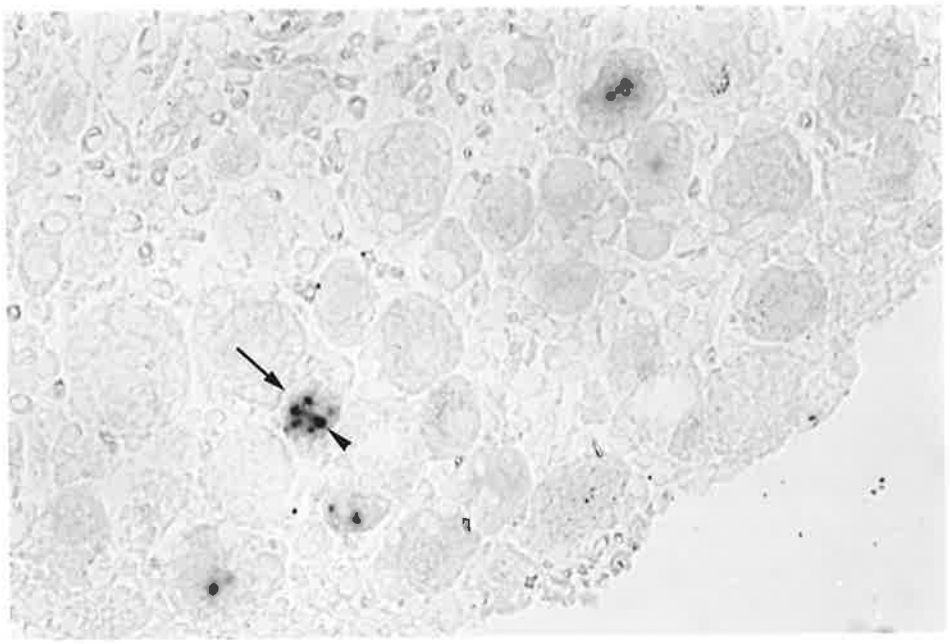
Figure 3.6. Photomicrographs illustrating the detection of minor LATs by ISH in sections (6 μm thick) of frozen drg from BALB/c mice 40 days after infection (a and c).

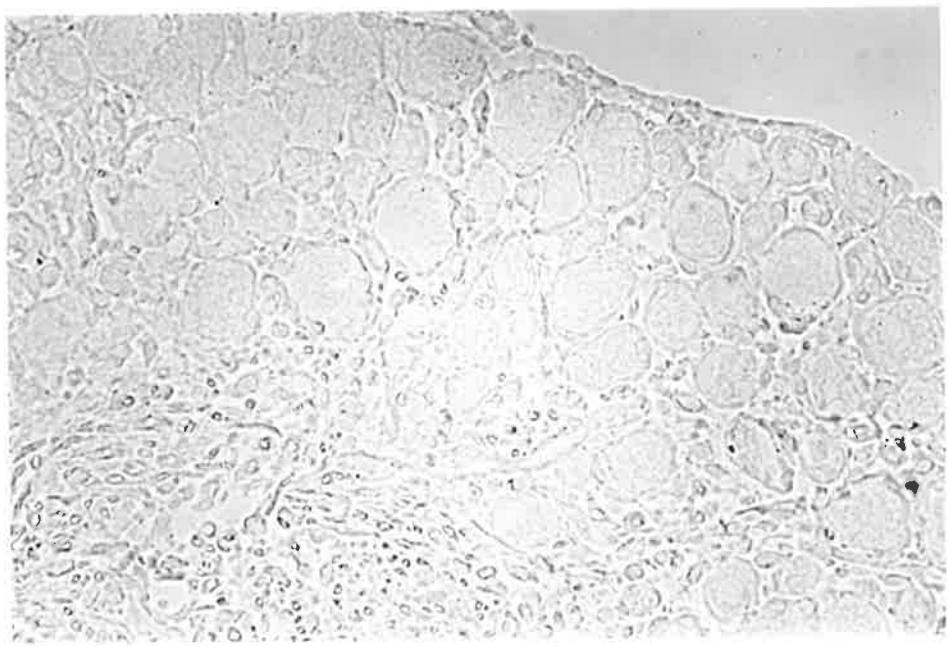
(a) Latently infected neurons (eg., arrow) showing intranuclear foci of hybridization (arrowhead) detected with a probe generated from pSLAT6.

(b) Profile of a ganglia from an uninfected BALB/c mouse illustrating lack of focal staining following hybridization with a minor LAT-specific probe generated from pSLAT6.

(c) Two latently infected neurons (arrows) showing intranuclear foci of hybridization (arrowheads) detected with a probe generated from pSLAT6.

Bound probe was detected with alkaline phosphatase-conjugated (a and b) or FITC-conjugated (c) anti-DIG Fab fragments. Magnification is (a and b) 390x; (c) 460x.





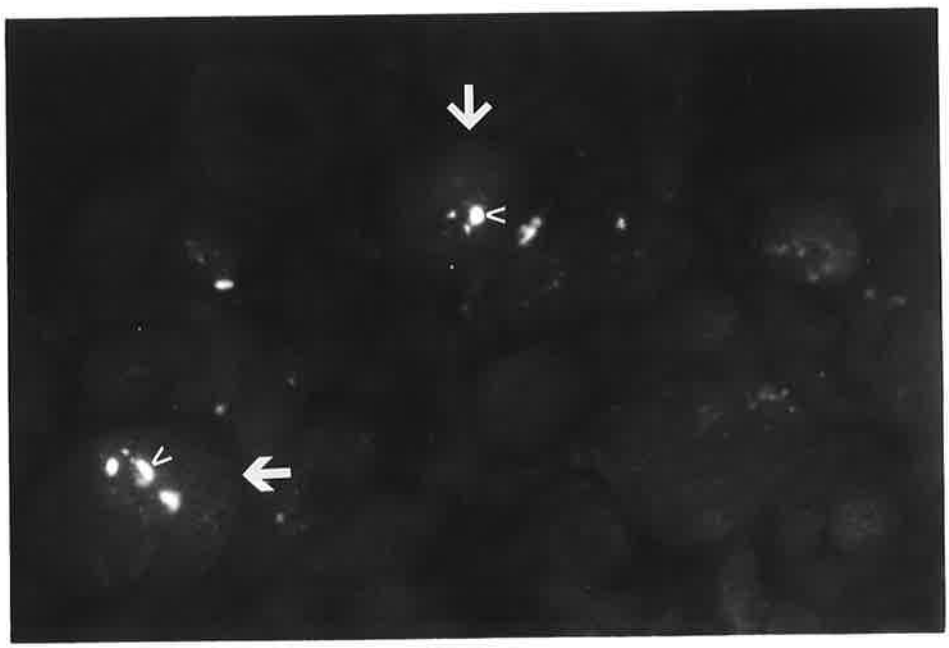
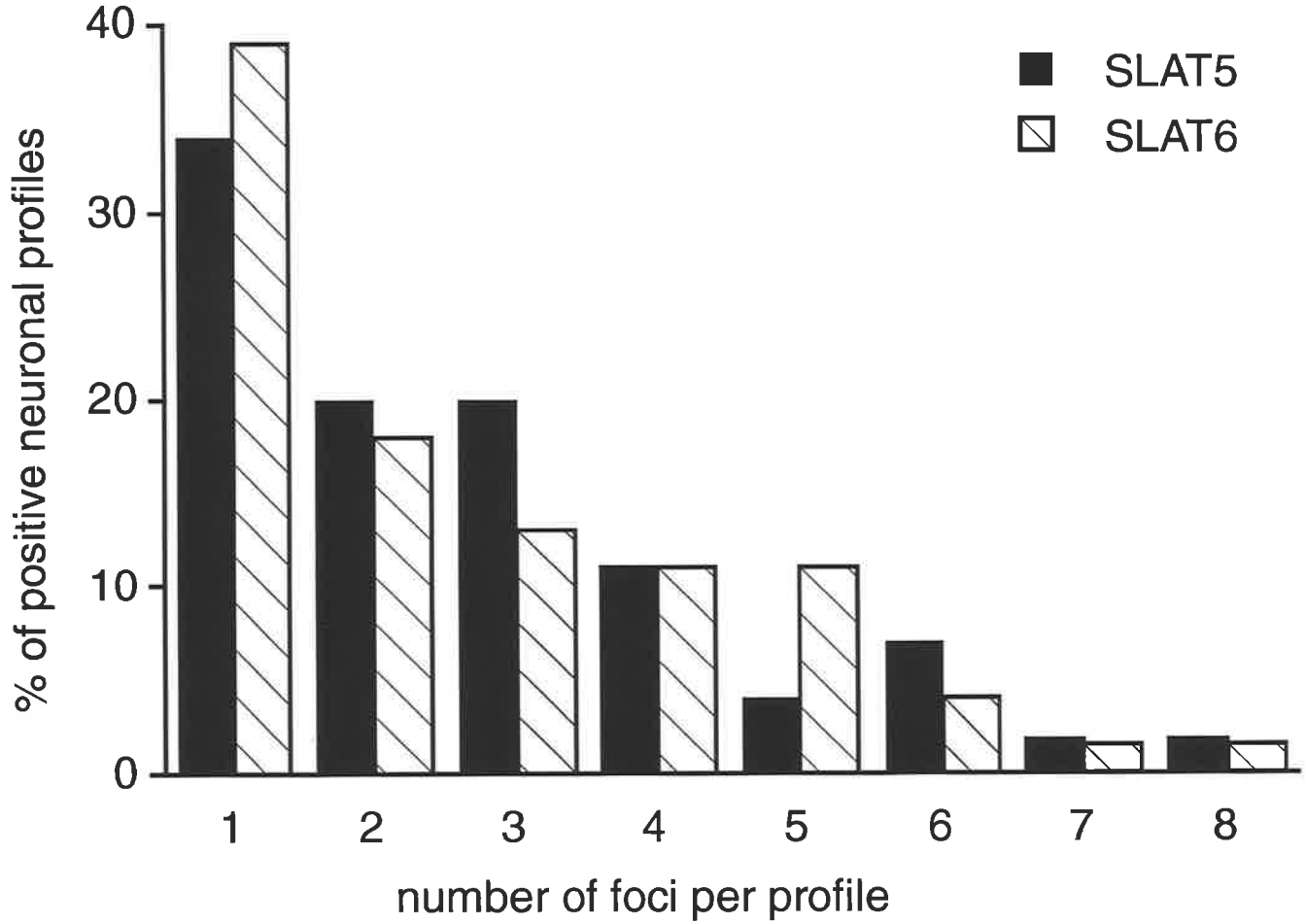


Figure 3.7 The number of minor LAT foci detected per LAT^+ neuronal profile with pSLAT5 or pSLAT6 probes, assessed in concurrent analysis of 183 ganglionic sections taken from eleven BALB/c mice 44 days after infection. The number of foci in a nuclear profile varied between 1 to 8 for each probe. The frequency with which each number of foci per profile occurred was plotted as a percentage (%) of total profiles counted per probe. On average 2.8 (pSLAT5) or 2.6 (pSLAT6) foci were detected per nucleus. In this tissue pSLAT5 probe stained on average 0.66 neurons per ganglionic section (99 sections in total) and pSLAT6 on average, 0.64 neurons (84 sections in total).


40
30
20
10
0
% of positive neuronal profiles
SLAT5
SLAT6
1
2
3
4
5
6
7
8
number of foci per profile

mice 44 days after infection. Probes generated from pSLAT5 detected 65 latently infected neurons in 99 ganglionic sections (0.66/section), with an average of 2.8 foci of hybridization per positive neuronal profile. Probes generated from pSLAT6 detected 54 latently infected neurons in 84 ganglionic sections (0.64/section), with an average of 2.6 foci of hybridization per positive neuronal profile. No apparent difference in the size of the foci generated by pSLAT5 and pSLAT6 probes was observed. Further, no difference was apparent in the distribution of foci detected with upstream versus downstream probes. Finally the numbers of foci per positive neuronal profile detected with pSLAT5 or pSLAT6 probes were similarly distributed (Figure 3.7).

Given that the size, number and distribution of foci detected by the two minor LAT probes were indistinguishable it was concluded that minor LATs arising from sequences between LAP1 and the 5' end of the major LAT region are most likely located within the same subnuclear domains as minor LATs arising from sequences downstream of the 3' end of the major LAT region.

3.3 INTRACELLULAR DISTRIBUTION OF LATS IN HUMAN TRIGEMINAL GANGLIA

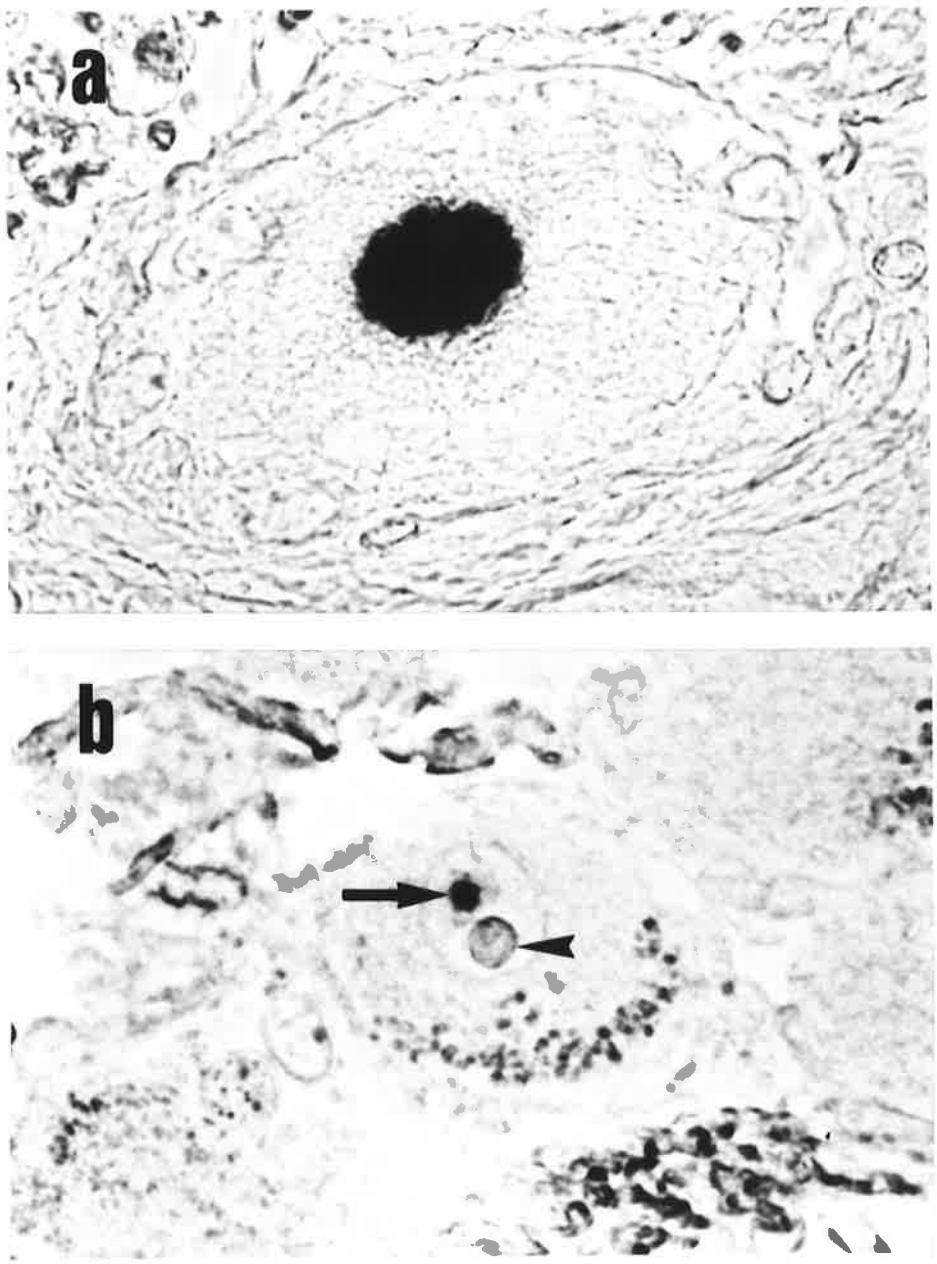
In the experiments described above the subcellular distribution of LATs was examined in dorsal root ganglionic tissues of experimentally infected mice. To determine whether the staining patterns observed for major and minor LATs were a feature of this animal model or characteristic of natural latent HSV infection, the intracellular distribution of LATs was examined in latently infected human trigeminal ganglia. Sections of PLP-fixed, HSV antigen negative, trigeminal ganglia from two seropositive cadavers were examined with probes generated from pSLAT4/E and pSLAT5. In agreement with the observations made

Figure 3.8. Light micrographs illustrating detection of LATs by ISH in sections (5 μm thick) of human trigeminal ganglia .

(a) A neuron showing diffuse nucleoplasmic staining (black) produced by LAT probes complementary to sequences within the major LAT region (generated from pSLAT4/E).

(b) A neuron (centre-field) containing a single intranuclear focus of intense hybridization (arrow) approximately half the diameter of the adjacent nucleolus (arrowhead).

Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments. Magnification is (a) 1200x; (b) 870x.


a
b

in latently infected mice, major LATs were distributed diffusely throughout neuronal nucleoplasms (Figure 3.8a) and minor LATs were located within sharply defined intranuclear domains (Figure 3.8b) in latently infected human trigeminal ganglia.

Thus it was concluded that the characteristic staining patterns of major and minor LATs in murine drg reflect the subcellular distribution of LATs in latently infected human ganglia.

3.4 SIMULTANEOUS DETECTION OF MAJOR AND MINOR LATS

In experiments above it was concluded that major and minor LAT sequences could be distinguished according to their spatial distribution in neuronal nuclei. Experiments using simultaneous detection of major and minor LAT sequences were done in order to determine if high and low abundance transcripts were generated within the same neuronal nuclei or synthesized by separate populations of latently infected neurons.

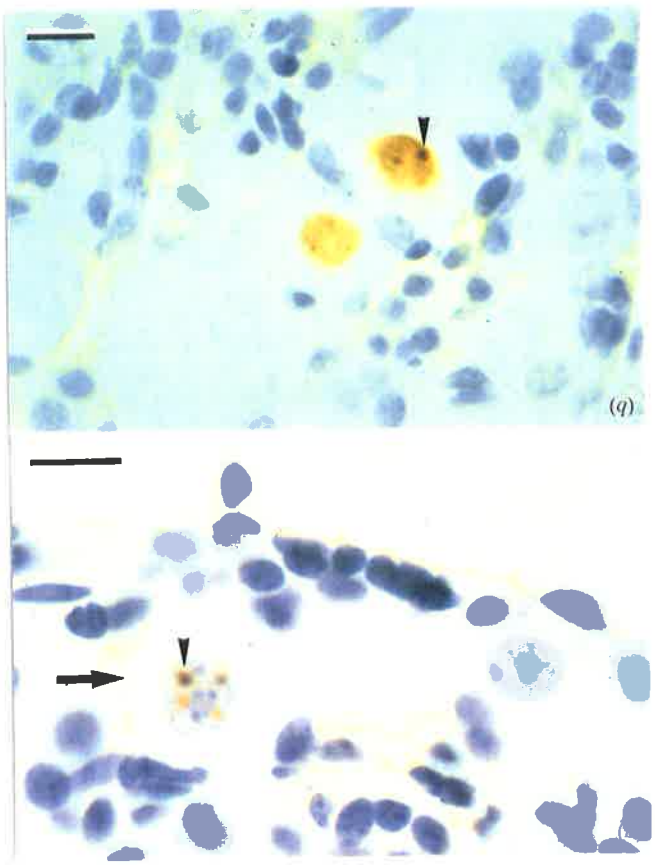
Ganglia removed from BALB/c mice 207 days after infection were hybridized with probes complementary to sequences spanning the 3' terminus of the major LATs (generated from pBSO templates). This probe gave rise to sharply defined foci of hybridization over a background of diffuse nucleoplasmic staining (Figure 3.9b) ie., the patterns characteristic of major and minor LAT sequences were superimposed. In the analysis of 61 positive neuronal profiles detected in 40 ganglionic sections (1.53/section), 33 (0.83/section) displayed only diffuse nuclear staining and 28 (0.7/section) showed foci of hybridization were detected in conjunction with diffuse nucleoplasmic staining (Figure 3.9b). No LAT^+ neuronal profiles contained foci alone. Using a truncated minor LAT-specific probe (pBSO/X) on the same tissue, 50 neurons containing foci were detected in 66 sections (0.76/section), indicating that diffuse staining did not interfere with detection of domains

Figure 3.9. Light micrographs of BALB/c ganglia illustrating detection of LATs by ISH.

(a) Section of a BALB/c ganglia 207 days after infection showing staining (brown) of intranuclear foci (arrowhead) within neurons (arrow) produced by hybridization with minor LAT-specific probe generated from pBSO/X.

(b) Sharply defined foci of hybridization (arrowhead) superimposed on diffuse nucleoplasmic staining (light brown), produced by a probe overlapping major and minor LAT sequences (generated from pBS0).

Binding of probes was detected using peroxidase-conjugated anti-DIG Fab fragments. Sections were counterstained with Mayer's haematoxylin (10 sec). Bar markers represent 25 μm.


(q)

containing minor LATs (Figure 3.9a).

Confirmation of the observations above was obtained using sections of latently infected BALB/c tissue (11 mice taken 44 days after infection) hybridized with a probe complementary to the major LAT region (pSLAT7), or a minor LAT-specific probe (pSLAT5) or a mixture of both. The sharply defined foci of hybridization typical of minor LATs (Figure 3.10a) and the diffuse staining typical of major LATs (Figure 3.10b) were also superimposed when a mixture of probes was applied (Figure 3.10c).

From these experiments it was concluded that (i) ~50% of major LAT^+ neuronal profiles also display foci minor LATs, thus indicating that high and low abundance transcripts may co-exist in latently infected neurons and (ii), the diffuse staining of major LATs in these experiments did not interfere with the detection of minor LAT domains.

3.5 DISCUSSION

High resolution, non-isotopic ISH was used to study the spatial organization of HSV-1 transcripts in primary sensory neurons of latently infected mice and humans. Previous studies, using radioisotopic ISH, have concluded that LATs are located predominantly in neuronal nuclei (eg., Stevens *et al*, 1987) but, the scatter of autoradiographic grains characteristically produced by radioactive probes limited the subcellular resolution of these experiments. In this study, major LATs were dispersed widely throughout the nucleoplasms of latently infected neurons but in addition, neurons in which the nuclei were stained intensely, weaker staining was often also observed in the perinuclear regions of the cytoplasm. This cytoplasmic staining may result from movement of RNA after sectioning or diffusion of the colour precipitate during signal development. Alternatively, this may

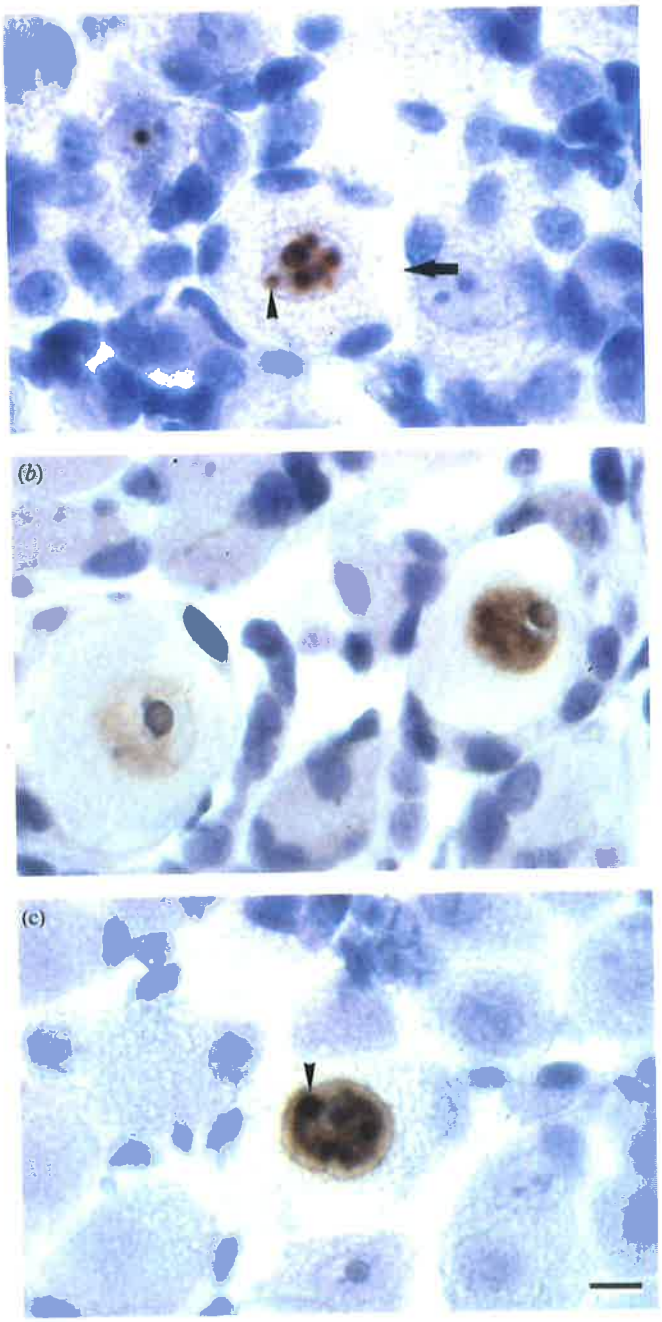
Figure 3.10. Light micrographs illustrating detection of LATs by ISH in 5 μm thick profiles of latently infected BALB/c ganglia from 11 mice 44 days after infection.

(a) Latently infected neuron (arrow) showing sharply defined intranuclear foci of hybridization (arrowhead) produced by a minor LAT specific probe generated from pSLAT5.

(b) Adjacent latently infected neurons displaying diffuse nucleoplasmic staining of varying intensity produced by a probe complementary to sequences within the major LAT region (generated from pSLAT7).

(c) Latently infected neuron showing foci (eg., arrowhead) superimposed on diffuse staining, produced by a mixture of probes (generated from pSLAT5 and pSLAT7).

Bound probes were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments. Sections were counterstained with Mayer's haematoxylin (10 sec). Bar marker represents 5 μm for (a) to (c).


(b)
(c)

represent migration of LATs to the cytoplasm during latent infection. In support of this hypothesis, major LATs have been detected in the cytoplasm, associated with polysomes, during acute infection in cell culture (N.W. Fraser, personal communication). Low levels of poly(A)$^+$ LAT-related RNAs have been detected by Northern blot during acute and latent phases of infection in cells *in vitro* and *in vivo* (Wagner *et al*, 1988a; Dobson *et al*, 1989; Zwaagstra *et al*, 1990) and it is possible that poly(A)$^+$ RNAs are transported to the cytoplasm during latent infection *in vivo*. Whether proteins are translated from cytoplasmic LATs at any stage during HSV infection of neurons requires further clarification. A protein product from ORF2 (which lies within the major LAT region) has been detected in neurons in an experimental model of latency *in vitro* (Doerig *et al*, 1991). This protein was larger than the predicted coding capacity of ORF2, suggesting that ORF2 may be an exon of a larger transcription unit. Although the *in vitro* latency system of Doerig *et al* (1991) suggests that RNA arising from part of the major LAT region can be transported to the cytoplasm and translated, it remains to be determined if these processes occur in latently infected neurons *in vivo*.

In striking contrast to the diffuse distribution of major LATs, minor LATs were not randomly distributed within neuronal nuclei but rather, they were concentrated into sharply defined foci. Indeed their intensely focal distribution rendered them readily detectable *in situ*, despite their low overall abundance in latently infected tissue (Mitchell *et al* 1990b). Further, foci of minor LAT hybridization were detected in a small proportion of infected neurons 5 days after flank inoculation. These cells may be equivalent to neurons reported by Speck and Simmons (1992) that enter latency without detectable immediate early gene expression.

High and low abundance transcripts could be readily differentiated according to their

spatial distribution. The staining patterns characteristic of major and minor LATs could be superimposed by using a mixture of probes, showing for the first time that these RNAs co-exist in latently infected neurons ie., they do not represent mutually exclusive patterns of transcription in different cell populations. The relative abundance of major and minor LAT sequences within the domains merits discussion. Foci were readily distinguished from a background of diffuse staining when a mixture of probes, or a probe spanning major and minor LATs was applied. In contrast discrete foci were not a feature of probes complementary to sequences wholly within the major LAT locus, even in weakly stained nuclei. Thus unlike minor LAT sequences, major LATs appear not to be concentrated in the focal domains. Identification of foci containing minor LAT sequences in excess of major LAT sequences is the first evidence supporting the possible existence of spliced transcripts *in vivo*. Whether these transcripts represent translatable poly $(A)^+$ mRNAs is not clear. Minor LATs were not detected within the cytoplasm of latently infected neurons and, at the time of writing this of thesis no HSV-1 encoded 'latent' protein has been identified within latently infected neurons *in vivo*. Should processed minor LATs be transported to the cytoplasm then they are present at concentrations below the level of sensitivity of the ISH protocol used. This situation would be analogous to that of the EBV RNAs encoding viral nuclear antigens, where the transcripts are readily detectable within cell nuclei, but not cytoplasms, during latent infection (Lawrence *et al*, 1989; Xing and Lawrence, 1991).

In mammalian cells a number of steps are required to generate translatable mRNA, including synthesis of the primary transcript, capping, hnRNP assembly, polyadenylation, splicing and transport to the cytoplasm (for review, see Nevins, 1983). Although a great deal of information exists regarding the biochemical processes involved in these steps, less is known about the distribution of the macromolecular complexes involved. However, in

recent years the application of high resolution ISH techniques has resulted in significant advances in our understanding of the functional organization of the nucleus. Mammalian nuclei have been shown to contain foci which are enriched in components of the pre-mRNA splicing machinery (Carmo-Fonseca *et al*, 1991 a&b). Further, poly(A)$^+$ RNA appears to reside within several nuclear foci which are considered to represent transcription and/or processing domains (Carter *et al*, 1991). Similarly, viral transcripts expressed in human immunodeficiency virus-infected cells (Lawrence *et al*, 1990) and EBV producer and non-producer cell lines (Lawrence *et al*, 1989; Xing and Lawrence, 1991) have been localized to foci or tracks, each focus being considered to be produced by a single transcriptionally active viral genome. In the experiments reported here, minor LAT-specific probes pSLAT6 and pSLAT5 respectively disclosed, on average, 2.6 and 2.8 intranuclear foci/LAT$^+$ neuronal profile. The average diameter of LAT$^+$ neuronal nuclear profiles (about 20 μm) was four times greater than the thickness of the sections (5 μm) and therefore there may be up to 10 to 11 foci, on average, per nucleus. This corresponds closely with the estimated number of viral genomes/LAT$^+$ cell in trigeminal (Rødahl and Stevens, 1992) or dorsal root ganglia (Simmons *et al*, 1992) of experimentally infected mice. Whether each minor LAT domain is associated with a single transcriptionally active viral genome could not be concluded from the experiments reported in this thesis and attempts by others to localize, at the subnuclear level, latent HSV DNA sequences by non-isotopic ISH have so far failed (B. Slobedman, personal communication).

The apparent size of the foci containing LATs (1-3 μm), whether stained by peroxidase-, alkaline phosphatase- or fluorocein-labelled antibodies were similar. The minor LAT domains are generally larger than the intranuclear domains of poly(A)$^+$ RNA or pre-mRNA previously visualized by fluorescence ISH in cultured cells ($\leq$1 μm; Carter *et al*, 1991; Wang *et al*, 1991; Jackson *et al*, 1993; Wansink *et al*, 1993). The size discrepancy may be

a reflection of the techniques used, or detection of different complexes. It is also possible that RNA processing compartments within the nuclei of neurons are much larger than those of HeLa cells. Further characterization of the macromolecular domains containing minor LATs, perhaps using electron microscopic ISH, will determine their true size.

It remains to be shown if minor LATs are mRNAs but if so, it is possible that the major LATs are introns. From analyses *in vitro* it has been suggested that major LATs are introns (Dobson *et al*, 1989; Farrell *et al*, 1991) although it remains to be demonstrated with certainty whether this is so *in vivo* during latency. Further experiments will be required to determine whether the diffuse nucleoplasmic distribution of major LATs is a consequence of their unusual stability or whether it is a general characteristic of intron sequences. Recently, Xing *et al* (1993) reported that intron sequences excised from neurotensin pre-mRNA were diffusely distributed within PC-12 neuroblastoma cell nuclei suggesting that diffuse intranuclear localization might be a general feature of intron sequences. The non-contiguous nature of major and minor LATs revealed here by their different intranuclear locations does not exclude the possibility that the 2.0 kb major LAT is a primary transcript initiated about 700 nucleotides downstream of LAP1, perhaps directed from LAP2 (Spivack *et al*, 1991; Nicosia *et al*, 1993; Goins *et al*, 1994).

In this study, high resolution ISH enabled the intranuclear locations of major and minor LATs to be differentiated, establishing a foundation for studying the synthesis, processing and transport of these molecules *in vivo*. It should now be possible to investigate the nature of those cellular products which associate with HSV-1 encoded LATs and thereby determine whether minor LATs are associated with previously characterized macromolecular complexes, such as those responsible for processing of pre-mRNA (Wang *et al*, 1991).

4. DISTRIBUTION OF NUCLEAR ANTIGENS IN CELLS *IN VITRO* AND *IN VIVO*

To examine the feasibility of determining whether HSV-1 encoded LATs colocalize with cellular macromolecular complexes of known composition and function, a method for the detection of nuclear antigens in murine dorsal root ganglionic neurons *in vivo* was developed. Antigens associated with three biochemically distinct regions of the nucleus were chosen for the initial analysis of neuronal nuclei. First, the distribution of splicing complexes was examined using antiserum directed against an antigen (designated Sm) contained in the core protein complex of the snRNPs associated with splicing of pre-mRNA (ie., U1, U2, U4, U5 and U6 snRNPs; Lerner and Steitz, 1979). The distribution of splicing complexes in neurons *in vivo* has not been described previously. Second, the localization of structural domains containing nucleolar snRNPs and fibrillarin was examined. Fibrillarin is a highly conserved (Jansen *et al*, 1991) 34 kD protein that interacts with multiple nucleolar snRNPs (Tyc and Steitz, 1989; Baserga *et al*, 1991) and is also found in coiled bodies (Ráska *et al*, 1991). The nucleolus is a distinct morphological and biochemical entity involved in the biogenesis of ribosomal RNAs and is perhaps the most striking example of the relationship between subnuclear localization and function. The function of coiled bodies is not clear (for review see section 1.5.2). Last, the distribution of specific regions of cellular chromatin were also examined using an antiserum directed against kinetochore proteins associated with chromosomal centromere regions (Moroi *et al*, 1980). Kinetochore protein complexes assemble at the centromeres and it is via these complexes that chromosomes are bound to the nuclear spindle at mitosis. Kinetochore proteins remain associated with the centromere during interphase (Moroi *et al*, 1981) providing a target for the immunohistochemical detection specific DNA sequences within cell nuclei. The distribution of centromeres within the cell nucleus is non-random and is

related to the state of differentiation of the cell (Spector, 1993).

High titre autoantibodies against a variety of nuclear antigens can be found in sera of patients with autoimmune disorders, in particular rheumatic diseases (Tan, 1989). Three human sera containing anti-nuclear antibody (ANA) activity were used for the immunohistochemical detection of these nuclear domains. Each serum used in this study contained antibodies specifically directed against antigenic determinants found in one of the nuclear domains discussed above. Human anti-nuclear antigen autoimmune sera have successfully been used by others in the examination of structure-function relationships in the cell nucleus (eg., Ráska *et al*, 1991; Carmo-Fonseca *et al*, 1991 a&b, 1993).

4.1 DETECTION OF NUCLEAR ANTIGENS IN CELLS *IN VITRO*

4.1.1 HEp-2 cells

The detection of nuclear antigens in HEp-2 cell monolayers was undertaken to provide a comparison for the examination of the distribution of nuclear antigens in neuronal cells *in vivo*.

Acetone-fixed HEp-2 cell monolayers were incubated with one of four human sera:

(i) *Lane*: autoimmune serum containing immunoglobulins that specifically bind Sm antigen enabling the detection of snRNP complexes associated with splicing;

(ii) *No*: autoimmune serum containing immunoglobulins that specifically bind nucleolar RNPs and fibrillarin enabling detection of nucleoli and coiled bodies;

(iii) *CC*: autoimmune serum containing immunoglobulins that specifically bind to chromosome centromere domains (kinetochores) enabling detection of defined regions of chromatin and,

(iv) *NHS*: pooled normal human serum, which does not bind specifically to nuclear antigens.

Bound antibodies were detected with rabbit anti-human antibodies conjugated to peroxidase or FITC.

Staining with Lane

In HEp-2 cell nuclei at interphase, *Lane* generated a speckled pattern consisting of numerous (greater than 20 per cell) small irregularly shaped granules ($\leq$1 μm in diameter) and weak diffuse staining, dispersed throughout the nucleus, with the exception of nucleolar regions (Figure 4.1a and 4.3 top). Further, 1 to 7 intensely stained spherical foci (~0.5 to 1 μm in diameter) were observed in the majority (~80%) of nuclei (Figure 4.1a and 4.3 top). *Lane* antiserum specifically binds to Sm antigen (G. Chang, personal communication) which is associated with snRNPs involved in splicing. The pattern observed is similar to the reported distribution of Sm and other factors associated with splicing in cells in culture, where it was concluded that staining represents the detection of snRNPs associated with perichromatin fibrils (ie., weak diffuse nucleoplasmic staining), interchromatin granules (staining of irregular shaped granules ~1 μm in diameter) and coiled bodies (intensely stained foci ~0.5 to 1 μm in diameter) (Elicieri and Ryerse, 1984: Leser *et al*, 1989; Carmo-Fonseca *et al*, 1991 a&b, 1993; Ráska *et al*, 1991; Spector *et al*, 1992). Thus, it was concluded that the staining observed in HEp-2 cells with *Lane* antiserum represents the distribution of macromolecular complexes responsible for splicing.

In contrast to the speckled staining generated with *Lane* antibody, staining was not observed in HEp-2 cell nuclei incubated with *NHS* (Figure 4.1d). In all subsequent experiments *NHS* treatment was included as a control for spurious antibody binding.

Staining with No

At interphase *No* disclosed 1 to 6 large granular domains and up to 10 small spherical foci (~0.5 to 1 μm in diameter) per nucleus (Figure 4.1b). The majority of the large granular domains were spherical (~2 to 6 μm in diameter) although, in some nuclei larger elongated domains were observed (Figure 4.1b). The large domains were rarely observed in contact with the nuclear envelope. Given that *No* detects nucleolar RNPs, it was concluded that the large domains (~2 to 6 μm in diameter) were nucleoli. The identity of the small spherical foci (~0.5 to 1 μm in diameter) requires further clarification but it is likely that most of the foci are coiled bodies. Staining of HEp-2 cells with *Lane* disclosed 1 to 7 coiled bodies per nucleus, which is fewer than the number of foci per nucleus (up to 10) detected with *No*, suggesting that the small spherical foci detected with *No* may be a mixture of coiled bodies and other small nucleolar domains. Alternatively, some coiled bodies may not contain snRNPs.

Staining with CC

Staining with *CC* in HEp-2 cell nuclei disclosed numerous discrete domains of less than 1 μm diameter (Fig. 4.1 c), dispersed throughout the nucleoplasm. In the majority of nuclei examined at least 20, and not more than 50, granules were observed. In each cell several of the domains were observed in close association with the periphery of nucleoli.

Given that *CC* contains antibody specific for antigens of the kinetochore complex, it was concluded that stained foci represent nuclear regions containing centromeric DNA.

Figure 4.1 Light micrographs illustrating the detection of nuclear antigens in HEp-2 cell monolayers.

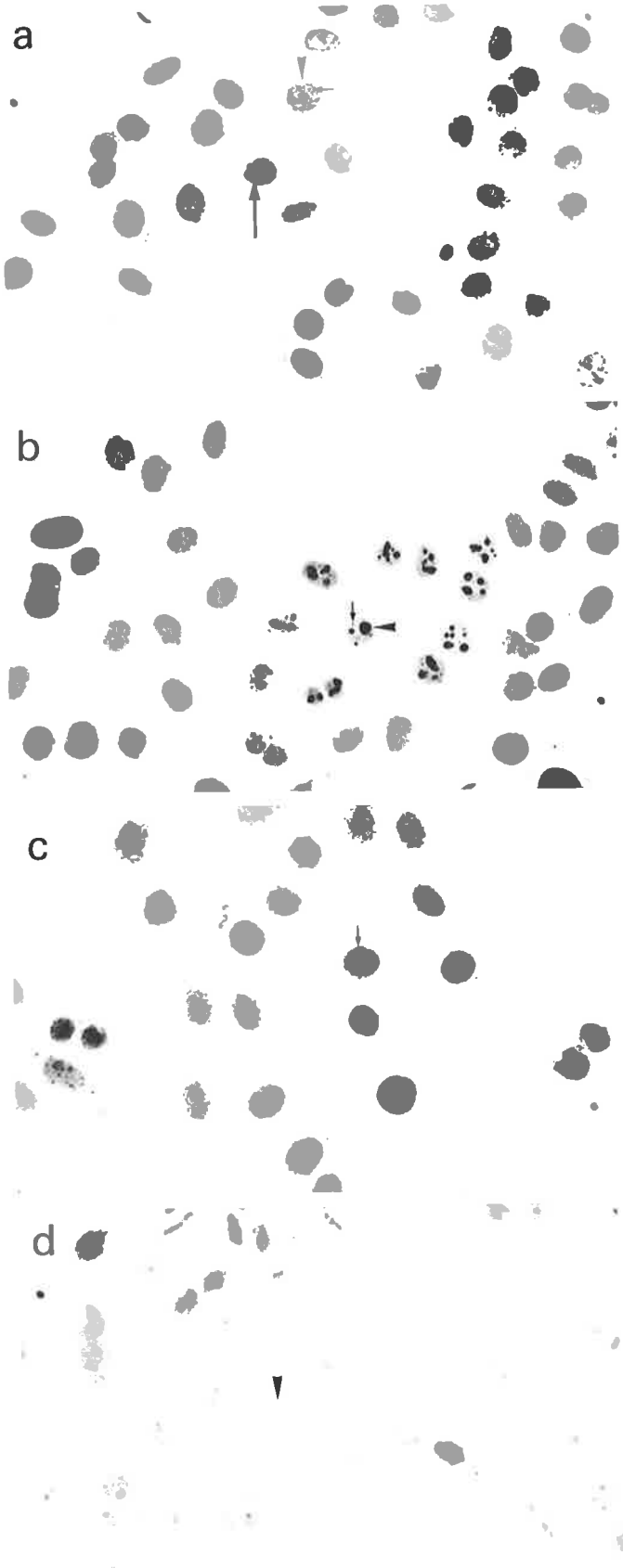
(a) Detection of splicing-associated snRNPs with *Lane* antiserum showing speckled staining of nuclei (arrowhead) plus intensely stained foci (eg. small arrow) above weak diffuse staining of the nucleoplasm with the exception of nucleolar areas (large arrow).

(b) Detection of nucleoli (arrowhead) and coiled bodies (arrow) with *No* antiserum in cell nuclei.

(c) Staining with *CC* showing the focal localization (arrow) of chromosomal centromeres (kinetochores).

(d) Cells showing a lack of nuclear staining (arrowhead) when incubated with *NHS*.

Bound antibody was detected with peroxidase-conjugated rabbit anti-human immunoglobulin. Magnification is 300x (a to d).


a
b
c
d

4.1.2 Neuro-2a cells

To determine the feasibility of using antibodies raised to human nuclear antigens for the detection of nuclear antigens in murine cells of neuronal origin, the distribution of nuclear antigens was examined in cell monolayers of murine neuroblastoma (Neuro-2a) cells.

Staining with *Lane* showed a speckled distribution in nuclei similar to that observed in HEp-2 cells. Compared with HEp-2 cells, Neuro-2a cells each contained less coiled bodies and further only ~50% of Neuro-2a nuclei (compared with ~80% of HEp-2 nuclei) contained coiled bodies. Coiled bodies of HEp-2 and Neuro-2a cells (not illustrated) were of a similar size (~0.5 to 1 μm).

Staining of Neuro-2a cells with *No* detected 1 to 6 nucleolar domains (~2 to 6 μm in diameter) per nucleus, resembling the staining seen in HEp-2 cells. In contrast, a maximum of three small foci ($\leq$1 μm in diameter) per nucleus were detected, which is somewhat less than the maximum number (10) observed in HEp-2 cells. Also the number of coiled bodies detected in the nuclei of Neuro-2a cells with *Lane* was similar (up to 3 per nucleus). Given that the number of coiled bodies per nucleus and the number of cells in a population displaying coiled bodies, is dependent on cell type (Ráska *et al*, 1991) it was concluded that the consistently lower number of coiled bodies in Neuro-2a nuclei was a characteristic of the cell line rather than a problem with the detection system.

Staining with *CC* produced discrete round foci ($\leq$1 μm in diameter), generally more than 20 per nucleus, similar to the staining pattern generated by *CC* in HEp-2 cells (not illustrated). No staining was detected when *NHS* antiserum was applied to Neuro-2a cells. All three specific antisera stained murine-derived neuroblastoma cells as strongly as HEp-2

cells leading to the conclusion that there is sufficient cross-species reactivity to enable specific detection of murine nuclear antigens with human antinuclear sera *Lane*, *No* and *CC*.

4.2 DETECTION OF NUCLEAR ANTIGENS IN MURINE DORSAL ROOT GANGLIONIC NEURONS *IN VIVO*

4.2.1 Detection of nuclear antigens in acetone-fixed frozen sections

Detection of the previously described nuclear domains was undertaken in acetone fixed frozen sections (6 μm thick) of drg from five BALB/c mice. Bound antibodies were detected with peroxidase- or FITC-conjugated rabbit anti-human immunoglobulins.

Detection of splicing-associated snRNPs

In sections of frozen drg, post-fixed in acetone, staining with *Lane* disclosed irregularly shaped nuclear speckles (granules ranging from ~1 to 4 μm in diameter) dispersed throughout neuronal nuclei (Figure 4.2a, 4.3 b&c). Further, fine punctate granules ($\leq$0.5 μm) were seen diffusely distributed throughout the nucleoplasm of neurons using the FITC detection system (Figure 4.3 b&c). Generally, 4 to 10 intensely stained speckles were observed per 6 μm thick profile. These domains were larger than the speckles and foci observed in HEp-2 and Neuro-2a cell nuclei using *Lane* antiserum (Figures 4.1a, 4.3a). The average diameter of neuronal nuclear profiles in acetone-fixed frozen tissue was about 15 μm, somewhat smaller than was observed in PLP-fixed tissues (~20 μm). Thus, 4 to 10 domains per profile represents, on average, 10 to 25 domains per neuronal nucleus. Similar results were obtained with peroxidase detection, although discrete granules in the diffuse nucleoplasmic staining were not as readily discernible with this enzymatic detection system

(not illustrated). Ganglionic tissue did not react with NHS (not illustrated).

Detection of nucleoli and coiled bodies

No detected up to 4 nucleolar domains (~2 to 6 μm in diameter) in neuronal nuclear profiles, although not all nuclear profiles contained such domains (Figure 4.2b). Further, neuronal profiles contained up to three small round foci ($\leq$ 1 μm in diameter) generally in close proximity to the periphery of nucleoli (Figure 4.2b). As in HEp-2 cells, the small domains detected in neurons were concluded to be small nucleolar regions and/or coiled bodies.

Detection of kinetochore complexes

Using a fluorescent antibody detection system, *CC* disclosed 5 to 10 discrete centromeric domains of $\leq$ 1 μm in diameter in neuronal nuclear profiles (Figure 4.2c). Centromeric domains were distributed throughout the non-nucleolar regions of the nucleus.

4.2.2 Distributions of splicing-associated antigens in paraformaldehyde-fixed tissues

To determine whether nuclear antigens could be detected in dorsal root ganglia fixed with paraformaldehyde, sections of

(i) PLP-fixed, wax embedded ganglia from 4 BALB/c mice and,

(ii) frozen ganglia from 5 BALB/c mice fixed after sectioning with 4% PB

were examined using *Lane* antiserum. Attempts to detect binding of antiserum in 5 μm sections of PLP-fixed tissue were not successful (not illustrated). Staining with *Lane* in 4%PB fixed frozen sections (6 μm thick) was observed in neuronal nuclei using peroxidase

Figure 4.2. Photomicrographs illustrating the detection of nuclear antigens in frozen sections (6 μm thick) of murine drg.

(top) Detection of snRNPs in neuronal nuclear profiles with *Lane* antiserum showing speckled staining of nuclei (arrow).

(centre) Detection of nucleoli (arrow) and coiled bodies (arrowhead) in neuronal nuclear profiles with *No* antiserum.

(bottom) Detection of chromosomal centromeres (kinetochores) (arrowhead) in profiles of neuronal nuclei (arrow) with *CC*.

Bound antibodies were detected with FITC-conjugated rabbit anti-human immunoglobulins. Magnification is (a) 510x; (b) 570x; (c) 1160x. Photographic exposure times were (a) 30 sec; (b) 3 min; (c) 2 min.

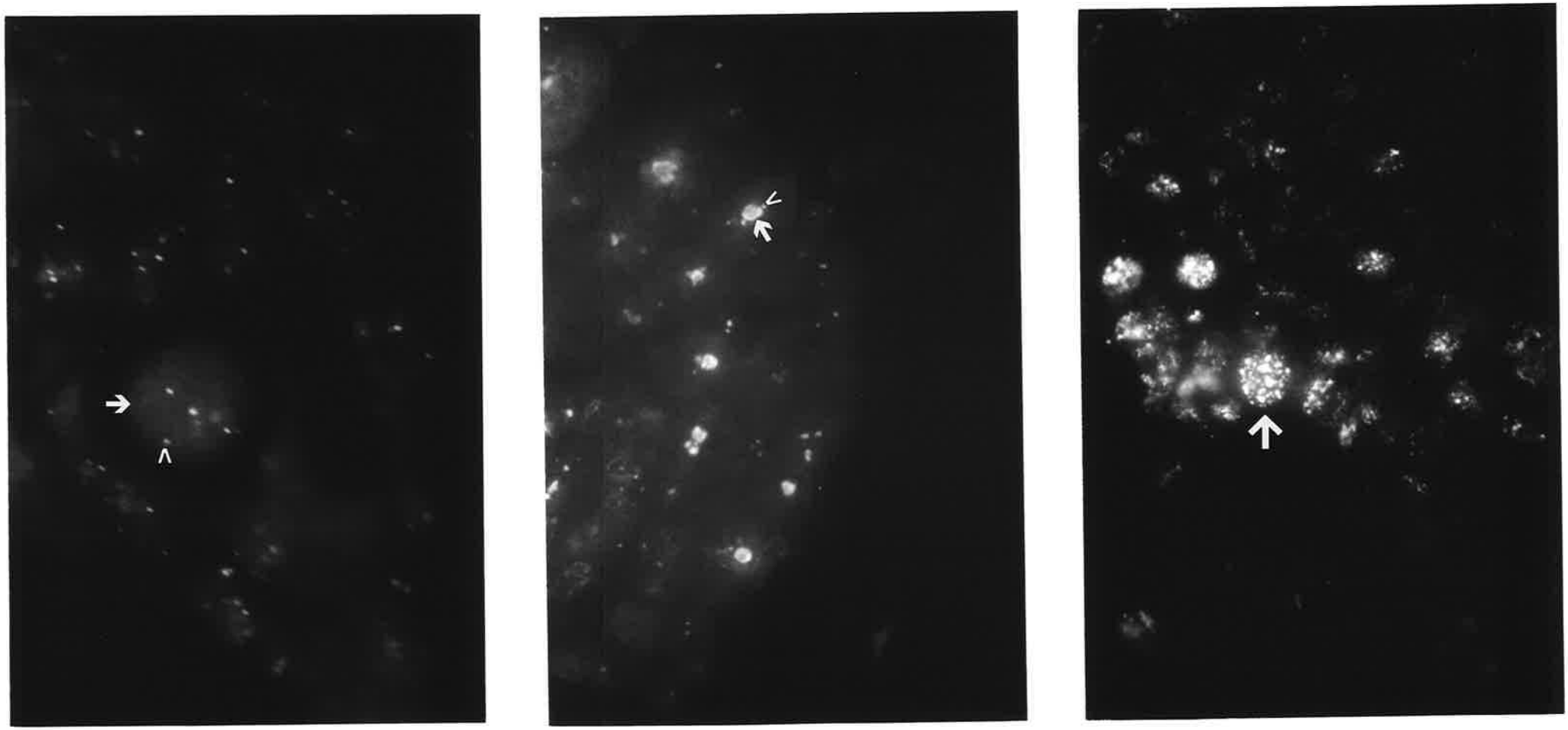
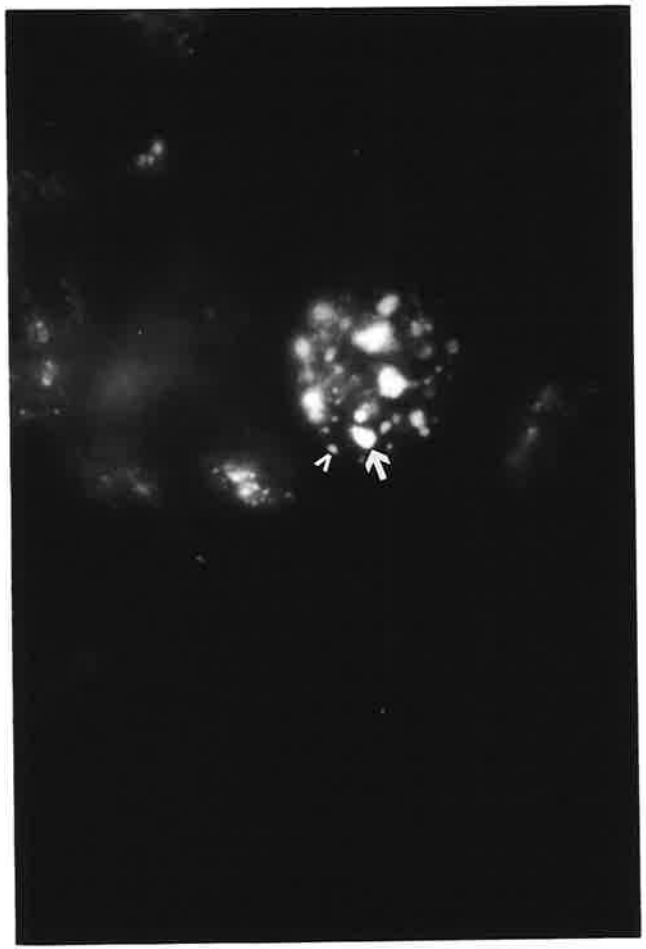


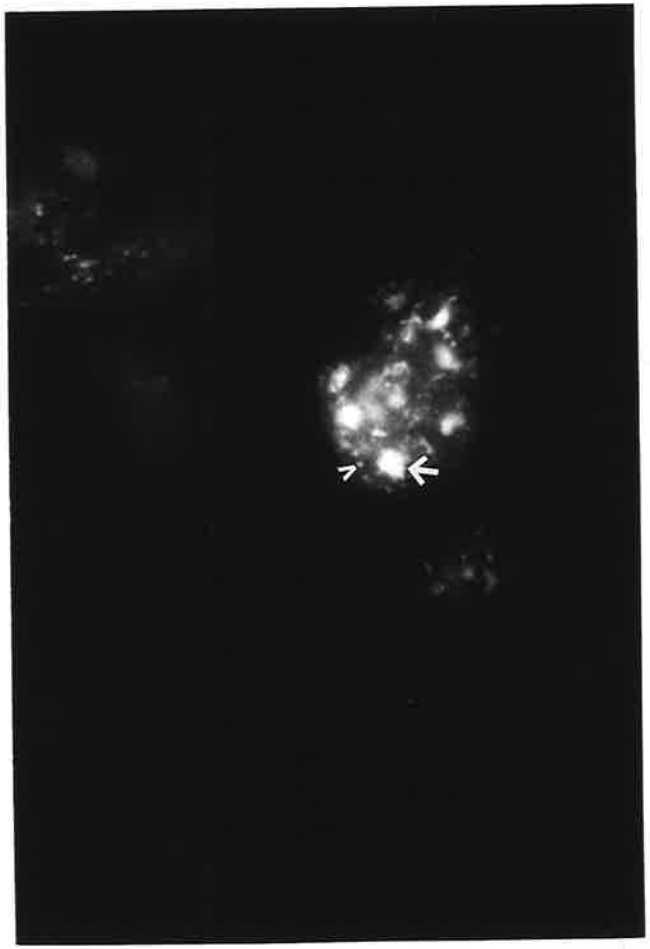
Figure 4.3 Photomicrographs illustrating the distribution of splicing associated snRNPs in HEp-2 cells and frozen sections (6 μm thick) of murine drg.

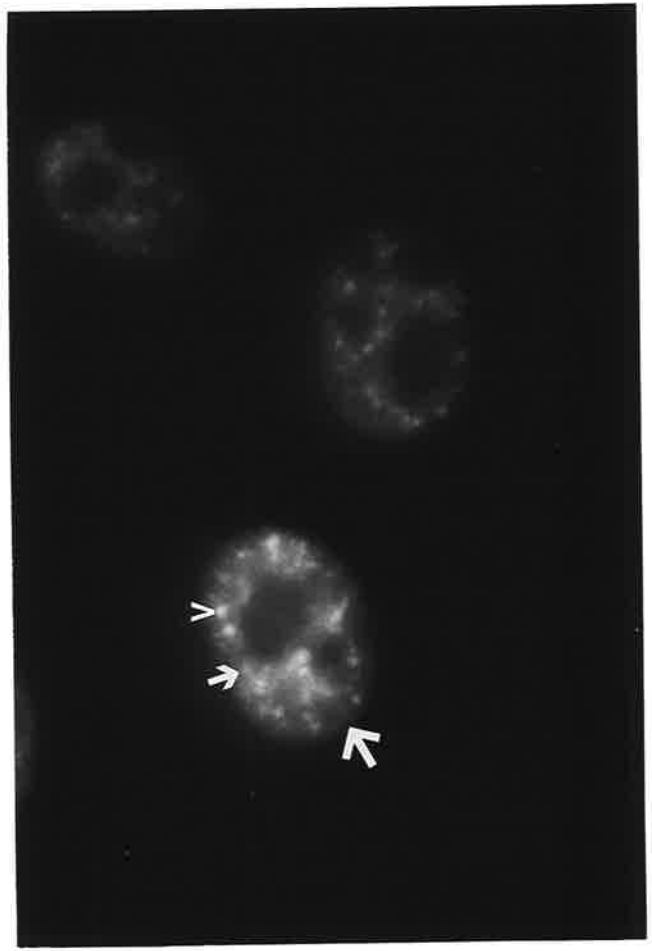
(top) Three HEp-2 cell nuclei showing staining pattern typical of snRNP detection with *Lane* antiserum ie., weak diffuse nucleoplasmic staining (large arrow), speckles (small arrow) and intensely stainied foci (arrowhead) with prominent sparing of nucleolar regions.

(centre and bottom) Two neuronal nuclear profiles displaying the staining pattern typical of snRNP detection with *Lane* antiserum ie., fine nucleoplasmic granules (arrowheads), large intensely stained foci (arrows) with weak staining of the nucleoplasm.

Bound antibodies were detected with FITC-conjugated rabbit anti-human immunoglobulins. Magnification is (a) 1400x; (b and c) 1200x. Photographic exposure times were (a to c) 30 sec.







development (not illustrated). However, staining was evenly distributed throughout the nucleoplasms of neurons with the exception of nucleolar areas and did not resemble the punctate staining previously observed in acetone-fixed sections of frozen drg. Further, foci of intense staining were not seen. Thus it appeared that the distribution of Sm in profiles of neuronal nuclei *in vivo* may have been altered by the use of 4%PB fixation in these experiments.

To determine whether binding of antibodies to nuclear antigens in frozen sections of drg could be detected if 4% PB fixation followed incubation of tissue with primary antiserum, acetone-fixed frozen sections (6 μm thick) of drg from 5 BALB/c mice were incubated with *Lane* antiserum, thoroughly washed in PBS (10 min) then refixed in 4%PB (15 min). Detection of bound human immunoglobulins resulted in a speckled pattern of staining similar to that previously seen in sections of drg treated with acetone only (section 4.2.1). That is, diffuse punctate staining of the nucleoplasm with sparing of nucleolar regions, plus up to 10 large (~1 to 4 μm in diameter) intensely stained foci, were seen in each nuclear profile (not illustrated). Thus treatment of sections with 4% PB prior to incubation with *Lane* antiserum compromised the detection of nucleoplasmic speckles in profiles of neuronal nuclei.

4.3 DISCUSSION

The feasibility of detecting macromolecular domains of known composition in profiles of murine dorsal root neurons was assessed. Nuclear antigens associated with splicing, nucleoli and chromosomal centomeres were targeted for investigation and detected using human autoimmune sera *Lane*, *No* and *CC* respectively. The staining patterns generated with these immune sera in HEp-2 cell monolayers have been characterized previously by

others (Immuno Concepts; G. Chang, personal communication). In the experiments reported here staining was initially done in HEp-2 and Neuro-2a cell monolayers to validate prior observations with these antisera and also to provide a comparison for staining in neuronal profiles. The intracellular localization of antigens associated with the domains examined in this study have also been characterized previously in HeLa cell monolayers (Moroi et al, 1981; Eliceiri and Ryerse, 1984; Leser *et al*, 1989; Carmo-Fonseca *et al*, 1991 a&b; Ráska *et al*, 1991; Spector *et al*, 1992).

The number of putative coiled bodies detected in HEp-2 cells by *No* was more than the number of coiled bodies previously detected by Carmo-Fonseca *et al* (1993) in HeLa cell nuclei, using an antibody to a protein (coilin) found only in coiled bodies. This suggests that either, HEp-2 cells contain more coiled bodies than HeLa cells or, the small fibrillarin domains in HEp-2 cells are a mixed population of coiled bodies and small nucleoli. Carmo-Fonseca *et al* (1993) showed definitively, in HeLa cell nuclei, that intensely stained spherical foci containing splicing associated snRNPs also contained coilin, suggesting that the intensely stained spherical foci detected in HEp-2 cells by *Lane* are coiled bodies. On this basis up to 7 coiled bodies were detected in HEp-2 cells with *Lane* antiserum, suggesting that the majority of the small domains detected by *No* must also have been coiled bodies. It has been shown the number of coiled bodies in a cell nucleus depends on cell type (Ráska *et al*, 1991) and that transformation of cells results in an increase in the number and relative size of coiled bodies per nucleus (Spector *et al*, 1992). Further, localization to coiled bodies of splicing factors, is dependent on continued transcription of pre-mRNA (Carmo-Fonseca *et al*, 1993).

In nuclei of Neuro-2a cells *No* detected fewer coiled bodies (up to 3 per nucleus), than in HEp-2 cell nuclei (up to 10). Dorsal root ganglionic neurons resembled Neuro-2a cells in

that they contained up to 3 putative coiled bodies often in close proximity to the nucleolar periphery. The size, distribution and number of the putative coiled bodies was consistent with the staining pattern generated by an antibody specific for coilin in 5 μm sections of neurons in rat brain tissue (Ráska *et al*, 1990) and rat neurons in primary culture (Carmo-Fonseca et al, 1993).

Staining of kinetochore complexes (by *CC*) should theoretically result in detection of each centromere region. In HEp-2 cells, *CC* detected between 20 and 50 discrete foci per nucleus. The variation in the number of centromeric domains detected per nucleus may reflect the aneuploid nature of this cell line or, indicate that the resolution of the stain was not sufficient to allow exact determination of numbers of foci. Centromeric domains in HEp-2 cells were discretely distributed throughout the non-nucleolar regions of the nucleus, although clustering of a few domains per cell was observed at the nucleolar periphery. Identification of clusters of centromeric domains at the nucleolar periphery in HEp-2 cells is in accordance with previous observations in a human lung carcinoma cell line in which centromeres were localized to the nuclear membrane or juxtaposed to nucleoli (Haaf and Schmid, 1989). In murine dorsal root ganglia, 5 to 10 foci were generally visible per neuronal nuclear profile. Given that the average size of neuronal nuclear profiles (6 μm thick) in these tissues was ~15 μm this corresponds to ~12 to 25 centromeric regions per nucleus. This suggests that either, some centromeric regions are in close association and appear as one domain on staining (as has been noted in some cell lines by Hadlaczky *et al*, 1986) or, the detection system used is not sufficiently sensitive to display all kinetochores (ie., 40 in diploid mouse cells). Billia and De Boni (1991) have recently reported that in differentiated dorsal root ganglionic neurons, centromeres were often associated with the nucleolus. In the profiles of neuronal nuclei examined in this thesis the position of the centromeres in relation to nucleolar regions was difficult to

ascertain. Dual colour fluorescent staining for nucleolar and centromeric domains in profiles of dorsal root ganglia may clarify this issue.

Staining of splicing snRNPs (by *Lane*) in profiles (6 μm) of murine dorsal root ganglionic tissues detected numerous large domains (~1 to 4 μm in diameter) plus smaller, punctate granules throughout the nucleoplasm of neurons. The large domains were on average, of greater size than the speckles and foci detected in HEp-2 and Neuro-2a cells. Carmo-Fonseca *et al* (1993) reported that immunohistochemical detection of Sm in primary cultures of rat hippocampal neurons detected several irregularly shaped speckles ~1 μm in diameter plus strong, widespread nucleoplasmic labelling. The speckles detected in rat hippocampal neurons *in vitro* were fewer in number and less well defined than the large snRNP domains detected (by *Lane*) in profiles of murine dorsal root ganglionic neurons.

The reason for the comparatively large size of snRNP-containing domains in murine drg neurons *in vivo* is not clear. It has been demonstrated that speckles of splicing-associated snRNPs coincide with nuclear regions enriched in interchromatin granules (Fakan *et al*, 1984; Spector *et al*, 1991). It has been proposed that interchromatin granules are sites of snRNP storage and/or assembly (Huang and Spector, 1991). Given that neurons *in vivo* are much larger than HEp-2 or Neuro-2a cells, it is possible that the large size of snRNP speckles in neurons reflects higher levels of transcription and greater requirement for splicing factors compared with smaller cells. A corollary of this may be that sites of snRNP assembly and storage contain greater amounts of snRNPs and consequently are larger. Interestingly, in HeLa cells grown at 39°C the distribution of Sm was predominantly concentrated to 20 to 50 speckles per nucleus and, a reduction in diffuse nucleoplasmic staining was noted (Carmo-Fonseca *et al*, 1993). The speckles seen at 39°C were, on average, larger and more intensely stained than the speckles observed in HeLa cells grown

at 37°C. These large speckles resemble those observed in neuronal profiles stained with *Lane*. Carmo-Fonseca *et al* (1993) concluded that the enlargement of snRNP domains in their experiments reflected the accumulation of snRNPs in storage sites resulting from reduced levels of transcription caused by thermal stress. Alternatively, the large size of snRNP-containing speckles in neuronal nuclei *in vivo* (detected with *Lane*) may be a reflection of the terminal differentiation of neurons. In studies of snRNP distribution in murine erythroleukemia (MEL) cells, which can be induced to differentiate in culture, Antoniou *et al* (1993) showed at late stages of differentiation large clusters of interchromatin granules develop (~1 to 4 μm in diameter), to which the majority of splicing snRNPs specifically concentrate. Since only a proportion of splicing snRNPs were associated with interchromatin granule clusters in undifferentiated cells, it was concluded that snRNPs migrate from other nuclear domains (including perichromatin fibrils and coiled bodies) into clusters of interchromatin granules at late stages of terminal erythroid differentiation (Antoniou *et al*, 1993). Thus it is possible that the large snRNP-containing domains detected in primary sensory neurons *in vivo* are large clusters of interchromatin granules. Monoclonal antibodies which specifically stain interchromatin granules are available and may be used to test this hypothesis.

At the initiation of this study of nuclear antigen distribution in neurons, the ultimate goal was to develop a method enabling the detection of specific nuclear domains in the nuclei of latently infected neurons *in vivo*. From the experiments reported in this chapter it is clear that accurate detection of nuclear domains in murine neuronal nuclei is possible. This establishes a foundation for analysis of the macromolecular complexes which colocalize with HSV LATs in latently infected neurons *in vivo*. Comparison of the distribution and size of the minor LAT foci described in Chapter 3 with the domains of splicing associated snRNPs detected with *Lane* indicates that minor LATs could colocalize with some of the

larger snRNP domains. The large snRNP domains may be clusters of interchromatin granules (Antoniou *et al*, 1993) and association of minor LATs with interchromatin granules would not be consistent with the hypothesis that minor LAT foci are sites of transcription given that, perichromatin fibrils are considered to be the sites of pre-mRNA synthesis (reviewed by Fakan, 1994). In other systems, staining for specific mRNA species within nuclei have led to the detection of the RNA in intranuclear foci or tracks (Lawrence *et al*, 1989; Huang and Spector, 1991; Xing *et al*, 1993). Further, Xing *et al* (1993) have demonstrated that RNA tracks overlap the position of the gene and are the site of intron removal. Colocalization studies in cultured cells have shown that tracks or foci of specific RNAs are near, or overlap, speckles enriched in splicing factors ie., interchromatin granules (Huang and Spector, 1991; Carter *et al*, 1993; Xing *et al*, 1993). The function of interchromatin granules in RNA biogenesis is not yet known and the significance of pre-mRNA association with such structures is not clear (Fakan, 1994).

The experiments reported in this thesis establish a foundation for the analysis of the nuclear factors that associate with HSV-1 LATs. It should now be possible to combine the detection of LATs, by ISH, with the immunohistochemical detection of nuclear antigens with defined functions, in frozen sections of murine drg which may provide valuable insight to the synthesis, processing and transport of LATs *in vivo*.

5 GENERAL DISCUSSION

5.1 LATENCY ASSOCIATED TRANSCRIPTION: FEATURES CONSERVED AMOUNG ALPHAHERPESVIRUSES

The latency associated transcripts of HSV-1 remain enigmatic. Although several potential functions for LATs have been postulated eg. protein encoding or antisense regulation of gene expression, their role during HSV-1 infection of neurons remains a mystery. Indeed from studies of LAT mutant viruses, it appears that the LATs are not an absolute requirement for establishment and maintenance of latency or for reactivation of latent virus. However, despite the lack of readily definable function, LATs are synthesized by all strains of HSV-1 studied and also by HSV-2. Further, restricted latency associated transcription, similar to that of HSV-1, is also observed during latent infection of neurons with two other alphaherpesviruses, BHV-1 and PRV. Transcription from latent BHV-1 genomes in trigeminal ganglionic neurons of cattle and experimentally infected rabbits generates two collinear RNAs about 0.77 and 1.16 kb in size (Kutish *et al*, 1990). Like the LATs of HSV-1, the latency related (LR) RNAs of BHV-1 are transcribed from a region of the genome which overlaps an immediate early gene, present in the opposite orientation on the complementary DNA strand (Kutish *et al*, 1990). Transcripts arising from latent PRV genomes consist of several partially collinear RNAs of about 8.5, 5.0, 2 and 0.95 kb in size (Cheung, 1989; Priola *et al*, 1990). PRV latent RNAs also map to a region of genome which overlaps the immediate early gene (IE180) and are transcribed in the opposite orientation, from the complementary DNA strand, to IE180 RNA. The 8.5 kb PRV RNA, termed the large latency transcript (LLT), is polyadenylated and has an ORF capable of encoding a 200 kDa protein. The other latent RNAs of PRV, like the HSV major LATs, do not appear to be polyadenylated. Further, as with HSV LATs the latent

transcripts of BHV-1 and PRV are predominantly observed in neuronal nuclei by ISH (Kutish *et al*, 1990; Priola *et al*, 1990). Transcription from latent VZV genomes differs from the alphaherpesviruses discussed above in that at least five discrete regions of the latent genome are transcribed (Croen *et al*, 1988). VZV may establish latent infection in satellite and possibly other supporting cells, rather than neurons and the different latent transcription profile is most likely a consequence of this.

In summary, despite any overall lack of sequence conservation between the transcribed regions of the alphaherpesviruses which establish latency in neurons (i.e. HSV-1, HSV-2, BHV-1 and PRV), all display restricted transcription from the latent genome, with the primarily nuclear localization of the resultant RNAs. Further, the position of the latency associated transcription unit in the viral genome is conserved ie., all LATs so far studied overlap one or more immediate early genes that are transcribed in the opposite orientation to LATs, from the complementary DNA strand. The positional conservation suggests that the latent RNAs of HSV may share a common function during infection of neurons. ORFs have been identified in latent RNAs but, because there is little sequence conservation between the ORFs of HSV-1, HSV-2, BHV-1, and PRV (Kutish, 1990; Cheung, 1991; McGeoch *et al*, 1991), it is unlikely that related proteins are responsible for any putative common function rather, LATs may interact directly with macromolecular processes. RNAs that function as transcriptional factors (Young *et al*, 1991), enzymes (Cech and Bass, 1986) or antisense RNA regulatory molecules (Eguchi *et al*, 1991) have been identified.

Given that HSV-1 LATs are found primarily in the nuclei of latently infected cells, the following discussion focuses on potential roles for nuclear RNAs. In particular the discussion addresses the functions of large nuclear polyadenylated transcripts, which may be of relevance to the role of the minor HSV LAT and the LLT of PRV. However it

should be noted that there may be more than one function for latency associated transcripts, one of which may be as messenger RNAs.

5.2 POTENTIAL FUNCTIONS OF THE HSV-1 LATS DURING LATENCY AND REACTIVATION

5.2.1 Does transcription from latent genomes facilitate rapid response to reactivation stimuli?

In recent years it has become apparent that nuclear processes are localized to domains within the nucleoplasm. For example, RNA synthesis is localized to several hundred discrete sites within the nucleus (Jackson *et al*, 1993; Wansink *et al*, 1993) and the association of splicing factors with such domains appears to be dependent on active transcription (Carmo-Fonseca *et al*, 1993). Further, Jiménez-Garcia and Spector (1993) have demonstrated that recruitment of transcription and splicing factors to sites of viral DNA occurs in response to initiation of viral transcription. It is conceivable that transcriptionally silent latent alphaherpesvirus genomes may, like inactive cellular chromatin, be spatially separated from factors associated with RNA biogenesis. In this scenario viral reactivation may be delayed while transcription factors are recruited to the viral genome. Continued transcription of spliced viral RNAs during latency would however, keep RNA transcription and processing factors associated with the viral genome, which may influence the rapidity of viral reactivation from latency. A conserved feature of the latent transcription units of HSV-1, HSV-2, BHV-1 and PRV is that they overlap one or more immediate early genes. In HSV-1, the LAT unit overlaps the gene for ICP0, a protein which may have a critical role in reactivation from latency (Leib *et al*, 1989a). Such positioning within the LAT unit may ensure that the gene for ICP0 is readily

accessible for transcription upon reactivation. It is interesting to note that most HSV-1 mutants which do not synthesize LATs during latent infection display reduced levels or delayed kinetics of reactivation (Javier *et al*, 1988; Leib *et al*, 1989b; Steiner *et al*, 1989; Rader *et al*, 1993) which may reflect time taken to recruit transcription and RNA processing factors to the latent viral genome.

5.2.2 Do minor LATs function as nuclear RNAs?

Due to their low abundance of in tissues latently infected with HSV-1, minor LATs have not been extensively characterized. Although potentially polyadenylated minor LATs have not been detected in neuronal cytoplasm. This may reflect the presence of low, undetectable levels of RNA in the cytoplasm or, a nuclear role for these RNAs. The localization of minor LATs to subnuclear domains (described in chapter 3) may reflect transcription sites or possibly sites of activity. Based on two examples described to date (discussed below), it appears that large polyadenylated nuclear RNAs may play novel roles in gene regulation.

Xist nuclear RNA

In mammalian females one of the X chromosomes is inactivated, thereby achieving gene dosage equivalence with males (Lyon, 1961). The molecular basis of X inactivation is not yet known but silencing of X-linked genes is at the level of transcription and is thought to involve a major cis-acting switch gene or inactivation centre (Xic). Not all genes are silent on the inactive X chromosome but the Xist gene, which maps to the Xic region, is so far unique in that it is transcribed exclusively from the inactive, rather than active X chromosome (Brockdorf *et al*, 1991). Xist RNA has been identified in female adult somatic cells of human and murine origin and is about 17 and 15 kb in size respectively

(Brockdorf *et al*, 1992; Brown *et al*, 1992). Although there is an overall similarity in the organization of the Xist genes of humans and mice, sequence analysis indicated a lack of any extended conserved ORFs. Further, the majority of transcripts are localized to the site of the inactive X chromosome. These observations suggest that Xist may function as structural RNA within the nucleus. Expression of Xist occurs early in developing female murine embryos, preceding X chromosome inactivation, and may be directly involved in the initiation and maintenance of X inactivation (Kay *et al*, 1993). Brockdorf *et al*, (1992) propose that Xist RNA may associate with unidentified nuclear factors and interaction of the resulting complex with the Xic chromatin region results in the inactivation of the X chromosome from which the RNA was transcribed. Alternately the Xist locus may be a chromatin organizer region and active transcription through this region induces changes to the chromatin structure at the Xic region to permit the binding of inactivating factor(s) (Brown *et al*, 1992).

Heat shock response omega RNAs of Drosophila

All organisms appear to have developed a specific and strikingly similar strategy by which to combat sudden changes in the extracellular environment, such as increases in temperature. This strategy, historically referred to as the heat shock response (hsr), involves the co-ordinated upregulation of the expression of a specific group of proteins whose collective function appears to be to protect the cell under stress and/or facilitate the restoration of basic metabolic pathways disrupted as a consequence of cellular stress. These hsr proteins (hsps) are synthesized from a group of genetic loci that share common promoter regulatory elements and the expression of hsps is tightly controlled, showing sudden upregulation in response to shock and rapid downregulation when the cellular stress is removed (reviewed by Welch, 1990).

Generally the active product of hsr loci are proteins but studies of the hsr in Drosophila has identified an unusual hsr locus, termed omega, the products of which appear to be regulatory RNAs. The hsr omega locus generates three partially collinear polyadenylated RNA transcripts of which the two largest, omega n (~10 kb) and omega pre-c (~2 kb) are nuclear. Although omega n and omega pre-c share the same initiation site they are products of alternate termination and further, they do not have a precursor-product relationship. The third transcript, omega c (~1.1 to 1.2 kb) is generated from omega pre-c by the removal of a single intron and is transported to the cytoplasm. These RNAs are constitutively expressed and are upregulated in response to cellular stress. The omega locus appears to be more sensitive to agents which influence cell physiology than the other major hsr loci. There is very little sequence conservation of the omega locus across Drosophila species but the structure of the locus, the shared promoter, 5' transcription start site, splicing sites and polyadenylation sites are all conserved.

Studies of the omega n transcript suggest that it has a novel regulatory role in the nucleus. By ISH omega n is observed in cell nuclei, concentrated at the transcription site with low levels dispersed throughout the nucleoplasm, excluding nucleoli (Hogan *et al*, 1994). Sequence analysis of the omega n RNA from different Drosophila species indicated that a major feature of the RNA was >8 kb of tandem repeats which make up the 3' end of the RNA which is not shared with the omega c RNAs. Although the sequence and size of the repeats is not well conserved across species a nine nucleotide sequence, AUAGGUAGG, is repeated about every hundred bases in all omega n RNAs and may serve as a protein binding site (Hogan *et al*, 1994). The amount of omega n is rapidly elevated in response to cellular stress and it has been proposed that this RNA may bind an abundant nuclear protein regulating the levels of the protein in the nucleus (Hogan *et al,* 1994). A rapid return to constitutive levels of omega n occurs following return of the cell to normal

conditions with the degradation of excess omega n apparently dependent on resumption of pol II transcription suggesting a novel intranuclear mechanism for RNA turnover.

Omega c contains a small ORF which appears to be translated, although only the first 4 amino acids encoded are conserved between Drosophila species. Further, no protein product from this ORF has been identified. In non-stressed cells omega c RNA is rapidly turned over in the cytoplasm but when translation is inhibited this RNA accumulates to high levels. Fini *et al* (1989) envisaged that the act of translation of omega c, rather than the generation of functional protein product is important and proposed that non-productive translation results in the degradation of omega-c RNA thus allowing the level of omega c to reflect the efficacy of protein synthesis in the cell. Thus the omega locus appears to provide a mechanism by which the levels of cellular translation may be monitored and, from the same transcription unit, a novel polyadenylated nuclear RNA is generated which may function to modulate levels of an abundant nuclear protein in response to cellular shock.

The above examples indicate that large polyadenylated nuclear RNAs may play important regulatory roles in the nucleus. Polyadenylation and completion of splicing are usually prerequisites for transport to the cytoplasm to occur and presumably in the case of Xist and omega n there is some overriding mechanism to retain these RNAs in the nucleus. Given that the 8 to 9 kb minor LAT of HSV is potentially polyadenylated and is apparently confined to the nuclei of neurons, possibly concentrated at sites of transcription, it is interesting to consider that the minor LATs may function in the nucleus in a manner similar to Xist or hsr omega n possibly to transcriptionally inactivate the viral DNA or, perhaps linking the latent virus to, or monitoring, the level of cellular stress.

The establishment and maintenance of HSV latency and reactivation involves the interplay of host cell and viral factors and it is likely that the LATs are an integral component of this relationship. Further understanding of the synthesis, processing and transport of these RNAs *in vivo* will be important in elucidation of the function of LATs. Insight to the interaction between LATs and neuronal macromolecular complexes, at the subcellular level, may be gained from colocalization studies utilizing a combination of the techniques developed in this project.

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