Expression from the herpes simplex virus type 1 latency-associated promoter in the murine central nervous system

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Herpes simplex virus type 1 (HSV-1) establishes latency in neurones of both the central (CNS) and peripheral nervous system and can be used to drive long-term expression of the lacZ reporter gene by insertion of an encephalomyocarditis virus IRES-linked gene 1.5 kb downstream of the latency-associated transcript (LAT) start site. However, the kinetics of LAT promoter (LAP) activity, and its ability to function in all neuronal types within the CNS has not been studied in detail. In order to address these issues, mice were infected via the ear pinna with $2 \times 10^6$ p.f.u. of either SC16-L$\beta$A, which contains an IRES-linked lacZ under the control of LAP, or SC16-C3b, which expresses LacZ under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter. Three to five animals from each group were sampled over a time-course from 5 days to 1 year post-infection (p.i.), and brainstem and spinal cord sections were examined histochemically for LacZ expression. We found that HCMV-IE promoter activity could be detected within distinct CNS regions from 5 to 15 days p.i. In contrast, LAP-driven LacZ expression was first detected at 7 days p.i. and persisted for at least 1 year. At times up to 34 days p.i., LAP activity was seen in similar regions of the CNS as those which were positive for HCMV-IE promoter activity during the acute stage of infection. After 34 days, however, the numbers of cells in which the LAP was active decreased and labelled motorneurones were predominantly detected in the facial and hypoglossal nuclei and occasionally also in the ventral spinal cord. These results suggest that following the establishment of latency in the CNS, the efficiency of long-term LAP-mediated gene expression may be influenced by the neuronal cell type in which latency is established.

Introduction

Herpes simplex virus type 1 (HSV-1) is able to establish lifelong latency within neurones. Following replication in epithelial cells at the point of contact, the virus infects nerve terminals which innervate the site of infection (reviewed by Roizman & Sears, 1996; Wildy et al., 1982), and is transported retrogradely along the axon to the neuronal cell body in the dorsal root (DRG) or trigeminal ganglion (Kristensson, 1970; reviewed by Enquist et al., 1998). Lytic replication in some of the infected neurones leads to the trans-synaptic spread of the virus to a reproducible series of sites within the central nervous system (CNS), where latency can be established (Knotts et al., 1973; Cook & Stevens, 1976; Engel et al., 1997; Cabrera et al., 1980; Ugolini et al., 1987, 1989; LaVail et al., 1990; Margolis et al., 1989; Deatly et al., 1988; Stroop et al., 1984). During latency the viral lytic promoters are transcriptionally silenced and viral gene expression, under the control of the latency-associated promoter (LAP), is restricted to the latency-associated transcripts (LATs) (Stevens et al., 1987; reviewed by Wagner & Bloom, 1997). However, it is not known whether all neurones, and particularly all CNS neurones, are capable of supporting transcription from the LAP, or whether the process is restricted to a particular subset of cells. In an earlier study, Kesari et al. (1996) used in situ hybridization (ISH) to map the establishment of latency following intracerebral injection of a neuroattenuated (ICP34.5 mutant) HSV-1. They reported that not all cells at the injection site expressed LATs, despite having access to the inoculum. We have previously reported that following injection of virus into the ear pinna, LAP-driven reporter gene expression can only be consistently detected in the facial and hypoglossal nuclei (Lachmann & Efstathiou, 1997), but it is unlikely that these are the only CNS nuclei...
accessed by replication-competent virus administered by this route.

To address this question we have used the mouse ear model of infection to study the establishment of latency in the CNS. The mouse ear is innervated by sensory nerves from the C2, C3 and C4 spinal levels, and motor neurones from the facial nucleus in the brainstem (Hill et al., 1975; Holstege et al., 1977). By infecting these neurones, HSV-1 can access the CNS via both the spinal cord and brainstem and pass transneuronally along the sensory and motor nerve pathways connected to them. As this route of inoculation leads to the infection of multiple types of CNS neurones which vary in size, morphology and function, we have been able to study the ability of the LAP to function in a wide variety of neuronal types. Using a recombinant HSV-1 (SC16-C3b) which expresses the lacZ reporter gene under the control of the cytomegalovirus immediate early (CMV-IE) promoter (inserted at the LAT locus), we have mapped out in detail the distribution and time-course of the acute virus infection in the CNS. With a second locus, we have mapped out in detail the distribution and time-course of the acute virus infection to study the establishment of latency in the CNS.

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### Methods

**Viruses.** Viruses were grown and assayed on Vero cells using Glasgow modified Eagle's medium supplemented with 10% foetal calf serum.

Construction of recombinant viruses was as follows. SC16-C3b contains the lacZ gene driven by the CMV IE promoter inserted into a 168 bp HpaI deletion (nucleotides 120301–120469) within the major LAT locus of HSV-1 strain SC16, as previously described (Lachmann et al., 1996). SC16-LlaA contains an encephalomyocarditis virus IRES linked to lacZ inserted into the same locus within major LAT, as described previously (Lachmann et al., 1997).

- **Mice.** Groups of 5–6-week-old BALB/c mice were infected with 2 × 10^6 p.f.u. virus, in 20 µl PBS, by subcutaneous injection into the left ear pinna. The number of animals sampled at each time-point is shown in Table 1. For all three viruses (SC16 wild-type, SC16-C3b and SC16-LlaA), 20–30% of the animals showed obvious signs of neurological impairment (such as limb paralysis) following infection, and so were euthanased and excluded from the study.

- **Tissue processing.** Animals were killed by terminal anaesthesia with pentobarbitone and perfuse-fixed transcardially with 20 ml of chilled 4% paraformaldehyde. The brain and cervical spinal cord were removed and post-fixed in 4% paraformaldehyde at 4 °C for 1 h. Sections from every animal were processed by β-galactosidase (β-Gal) histochemistry, and in some animals alternate sections were processed by immunohistochemistry using an antibody to β-Gal.

- **β-Gal histochemistry.** The brain was embedded in 4% LGT agarose. Sections (100 µm) were cut with a vibratome and collected in detergent solution (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM MgCl₂, in PBS) in 24-well plates and incubated at 4 °C. After 2 h the detergent was replaced with X-Gal solution (1 mg X-Gal per ml of detergent solution, with 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide) and incubated at 37 °C for 2–12 h. The sections were then rinsed in three changes of PBS, mounted on polylysine-coated slides and air-dried. The tissue was then defatted in xylene, rehydrated through graded ethanols and counter-stained with neutral red.

- **Immunohistochemical detection of β-Gal.** Following fixation, the brains and spinal cords were equilibrated in 25% sucrose for 24 h. Frozen sections (60 µm) were cut using a sledge microtome. Alternate sections were taken for β-Gal histochemistry and immunohistochemistry, respectively. Sections taken for immunohistochemistry were immersed in 3% hydrogen peroxide, 10% methanol solution for 5 min to quench endogenous peroxidase activity, rinsed with three 10 min changes of TBS (0.05 M Tris–HCl, 0.15 M NaCl, pH 7.4) and blocked for 1 h with 3% normal goat serum (NGS) in 0.2% Triton-X in TBS. They were then incubated overnight with a rabbit IgG to β-Gal (ICN/Cappel cat. no. 55976) diluted 1 in 6000 in 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.4 and blocked for 1 h with 3% normal goat serum (NGS) in 0.2% Triton-X in TBS. The secondary antibody was a biotinylated goat anti-rabbit antibody diluted 1 in 200 in TBS, 1% NGS. β-Gal-positive cells were revealed using DAKO streptABComplex/HRP (Dako – K 0377) with 0.6 mg/ml DAB containing 0.03% hydrogen peroxide.

- **In situ hybridization.** Brains and spinal cords were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin, and 5 µm sections were cut using a microtome. Every 20th section was mounted onto a 3-aminopropyltriethoxysilane-coated glass slide. ISH was carried out using a LAT-specific probe as described previously (Arthur et al., 1993).

- **Plotting of labelled cells.** Tissue from an infected animal was sectioned, mounted and counterstained as described above. Drawings of the sections (made with a camera lucida) were transferred to CorelDraw 8.0 using a Wacom A3 digitizing plotter, and were labelled according to the atlas of Paxinos & Watson (1986). Labelled cells were then plotted onto copies of each of the drawings with CorelDraw.

### Results

#### CMV-IE promoter – kinetic and anatomical analysis

To trace the spread of HSV-1 following ear infection, mice were infected with 2 × 10^6 p.f.u. of virus SC16-C3b (containing

<table>
<thead>
<tr>
<th>Time-point (days p.i.)</th>
<th>SC16-C3b</th>
<th>L/A</th>
<th>SC16</th>
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<td>34</td>
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<td>1 (40 days)</td>
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<td>1 year</td>
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**Table 1. Summary of the number of animals studied with each virus over time**
the CMV IE promoter driving lacZ) and animals were sampled at 5, 7, 11, 15 and 21 days post-infection (p.i).

**Spinal cord**

**5 Days.** In the spinal cord the majority of the labelling was found on the infected side (left), in and around the dorsal columns, Lissauer’s tract, and the dorsal horn grey matter, from the level of C3 upwards (Fig. 1). At this time, a column of labelled fibres and glial cells was seen in the left dorsal root entry zone and dorsal column, extending rostrally to the brainstem. In the spinal grey matter, infected neurones were found in the superficial (II, III, IV) and deeper Rexed laminae (V, VII and X). Longitudinal spinal cord sections showed that these labelled cells formed a continuous column in the dorsal horn, stretching from approximately the level of C4 to C2 (Fig. 1).

**7 Days.** Labelled cells were found at the same locations as seen on day 5 and also in small numbers of motoneurones in the ventral horn (see Fig. 4 A). In some cases the virus had also spread to a few neurones in the same regions on the contralateral side of the spinal cord.

**11 and 15 Days.** By this time the majority of the labelled cells in the spinal cord had disappeared, leaving behind severe inflammation and vacuolation which, in some places, involved the entire dorsal column and dorsal horn on the left. The inflamed areas were swollen and showed a dense cellular infiltrate.

**21 Days.** No cells were labelled by the CMV-IE promoter and the spinal cord contained only regions of inflammation where virus replication had occurred.

**Brainstem**

The distribution of labelling seen in the brainstem over time is presented in Fig. 2. For clarity the combined results of two animals each from the 5, 7 and 15 day time-points are shown, since at day 11 the distribution was very similar to that seen at

Fig. 1. Spinal cord architecture and the spread of HSV-1 through the spinal cord grey matter. Cutaneous sensory afferents enter the spinal cord via the dorsal roots and send collaterals to the brainstem (in the dorsal columns) and also to specific laminae in the dorsal horn, as illustrated in red and green in (A). The cells in the dorsal horn that receive the laminar projections are organized into a series of overlapping cell columns that extend longitudinally along the spinal cord. This cell column corresponds to the blue region in (B), which represents a longitudinal section through the dorsal spinal cord. Five days after ear pinna infection with virus SC16-C3b (CMV-IE promoter driving lacZ), neurones are specifically labelled in this cell column, as seen in (C), which is a longitudinal section through the dorsal spinal cord. Transneuronal spread of the virus up and down the cell column brings the virus into contact with the axon terminals of DRG neurones at higher and lower ganglionic levels of the cord, permitting the establishment of latency at these sites.
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Fig. 2. For legend see facing page.
HSV-1 LAP activity in the mouse CNS

Fig. 2. The distribution of cells labelled in the brainstem by the CMV-IE promoter at 5 (green dots), 7 (red dots) and 15 days (blue dots) p.i. The drawings show a caudal to rostral series through the brainstem at 300 µm intervals.

Abbreviations used in anatomical diagrams:

4th – Fourth ventricle
5m – Trigeminal motor nucleus
5n – Trigeminal nerve root
5s – Spinogenu trigeminal nucleus
5t – Trigeminal tract
6 – Abduncus nucleus
7 – Facial nucleus
7g – Facial nerve genu
7n – Facial nerve root
10 – Vestibular motor nucleus
12 – Hypoglossal nucleus
A – Aqueduct
Amb – Ambiguous nucleus
Chm – Corpus middle geniculatum laterale
CCx – Cerebral cortex
CoN – Ventral cochlear nucleus
Cod – Dorsal cochlear nucleus
DBC – Decussation of brachium conjunctivum
DCN – Deep cerebellar nuclei
dRF – Dorsal reticular formation
dMR – Deep mesencephalic reticular formation
DT – Dorsal tegmental area
(Ext) CuN – (External) Cuneate nucleus
GRC – Gissengrauel cell groups
GrN – Gracile nucleus
IC – Inferior colliculus
Icp – Inferior cerebellar peduncle
ION – Inferior olivary nucleus
LDT – Lateral dorsal tegmental area
LI/II – Lateral lemniscus
LRN – Lateral reticular nucleus
ml – Medial lemniscus
NRTP – Nucleus reticularis tegmenti pontis
NTS – Nucleus tractus solitarius
PAG – Periaqueductal grey
PrR – Parvicellular reticular formation
Py – Pyramidal decussation
PyX – Pyramidal decussation
R – Raphe nuclei
RB – Reticularab nucleus
RN – Red nucleus
SC – Superior cerebellum
scp – Superior cerebellar peduncle
SN – Substantia nigra
SON – Superior olivary nucleus
V1 – Lateral (Deiter’s) vestibular nucleus
Vm – Medial vestibular nucleus
Vs – Superior vestibular nucleus
Vsp – Spinal vestibular nucleus
VRf – Ventral reticular formation
VT – Ventral tegmental area

Day 15, and at day 21 only one or two labelled cells were detected in the brainstem of each animal.

5 Days. The distribution of labelled cells seen in the brainstem at 5 days is shown in green in Fig. 2. The mean cell count per brainstem (from the level of the pyramidal decussation to the superior colliculus) was 220 (range 188–253, n = 3). The labelled cells were predominantly found on the left side, scattered around the facial nerve and in the medial half of the facial nucleus. In some animals small numbers of labelled motorneurones were also seen in the contralateral (right) facial nucleus, possibly due to crossed innervation of the ear from the contralateral side. The rostral limit of the infection was at the level of the locus coeruleus, which was consistently labelled on both sides of the brainstem. In the caudal medulla, labelled neurones and fibres were found in the left cuneate nucleus, consistent with ascending spread of the infection from the left spinal cord dorsal column, and a small cluster of labelled cells was detected in the reticular formation adjacent to the left lateral reticular nucleus. Small numbers of labelled cells were also found in the Raphe nuclei at all levels of the brainstem.

7 Days. The distribution of labelling remained grossly the same, though the zones of infection were larger, especially contrateraterally, and the virus had spread to a number of additional sites in the brainstem. The mean cell count per brainstem was 194 (range 70–280, n = 3) and by 21 days it was only 1 or 2. In Fig. 2, the distribution of labelled cells seen at 15 days is shown in blue. By this time-point, in some animals, the virus had also spread to the dorsal reticular formation, the pedunculopontine nucleus and the inferior and superior colliculi. Significantly, the brainstems of animals sampled at 15 and 21 days all contained variable-sized zones of inflammation and vacuolation. The tissue destruction was most marked in regions where the labelling had been found to be especially dense in animals sampled at earlier time-points, particularly in the reticular formation around the left facial nerve and also in the medial part of the left facial nucleus, which was completely destroyed in some animals.

LAT promoter

To identify the sites of LAP activity in the brainstem and spinal cord, animals were inoculated with 2 x 10⁶ p.f.u. of

LDT – Lateral dorsal tegmental area
LI/II – Lateral lemniscus
LRN – Lateral reticular nucleus
ml – Medial lemniscus
NRTP – Nucleus reticularis tegmenti pontis
NTS – Nucleus tractus solitarius
PAG – Periaqueductal grey
PrR – Parvicellular reticular formation
Py – Pyramidal decussation
PyX – Pyramidal decussation
R – Raphe nuclei
RB – Reticularab nucleus
RN – Red nucleus
SC – Superior cerebellum
scp – Superior cerebellar peduncle
SN – Substantia nigra
SON – Superior olivary nucleus
V1 – Lateral (Deiter’s) vestibular nucleus
Vm – Medial vestibular nucleus
Vs – Superior vestibular nucleus
Vsp – Spinal vestibular nucleus
VRf – Ventral reticular formation
VT – Ventral tegmental area
Fig. 3. For legend see facing page.
SC16-LβA (in which LacZ expression is driven by the LAP) in the left ear pinna. Animals were sampled at 7, 11, 15, 21 and 34 days p.i. and a second group of seven animals was examined at 1 year p.i. In animals infected with SC16-LβA the labelled cells all appeared to be neuronal, although in brain regions where there had been obvious cellular destruction and inflammation, such as in the spinal cord dorsal columns and along the facial nerve course, non-neuronal cells around the sites of infection sometimes produced a weak pattern of staining. These cells were most likely phagocytic and stained positively because they had engulfed LacZ-positive neuronal debris.

Spinal cord – LAT promoter. The pattern of LAP labelling in the spinal cord from 7 to 21 days was identical to that seen acutely with the CMV-IE promoter (see Fig. 4 A, B). Labelled neurones were present in the dorsal horn, around the central canal, and in the ventral horn. Labelled fibres of latently infected DRG cells could be seen running in the dorsal roots, dorsal horn and left dorsal column. At 34 days, however, most of the labelled cells had disappeared and the only positive cells remaining were motorneurones in the ventral horn.

Brainstem – LAT promoter. Over the 34 days following infection, labelled cells were detected in the brainstem at all the same sites that were labelled by the CMV-IE promoter acutely, including the locus coeruleus, periaqueductal grey, the reticular formation, the perihypoglossal, trigeminal, facial and hypoglossal nuclei (Fig. 4). The anatomical distribution of labelling seen in the brainstem at successive time-points is presented in Fig. 3. For the first 21 days the numbers of labelled cells increased in each brain region; at 7 days the mean labelled cell count per brainstem was 468 (range 409–526, n = 3), at 15 days 920 (range 329–1885, n = 3) and by 21 days (when cell labelling by the CMV-IE promoter had ceased) 1083 (range 1029–1136, n = 3). However, by 34 days p.i. the numbers of labelled cells were greatly reduced. In most of the CNS regions that had contained labelled cells at earlier time-points (perihypoglossal nucleus, the locus coeruleus, periaqueductal grey), only a few weakly staining cells were present (two of three animals). The majority of the labelled cells were motoneurones in the facial (three of three animals) and hypoglossal nuclei (two of three animals), the trigeminal motor nucleus (Vm) and the trochlear nucleus (IV) (one of three animals). In all of these animals, even at this time-point, there were still clear signs of inflammation, suggesting a persistent immune response, perhaps to the LacZ expression driven by the LAP.

At the 1 year time-point, about half of the animals sampled had labelled cells in the brainstem (four of seven). The labelled cells were predominantly motoneurones in the facial and the hypoglossal nuclei, but the odd positive cell was also found in some of the other regions which we found to have been infected acutely, such as the dorsal column nuclei.

Comparison of sites of CMV promoter and LAT promoter labelling

To demonstrate that the LAP is functional in the same regions of the brainstem as those that are infected acutely (as shown by CMV-IE activity) we superimposed plots of the distributions of CMV-IE-labelled and LAP-labelled cells. There is a very close correspondence between the regions showing CMV-IE activity and regions showing LAP activity for all the time-points studied. As an example, Fig. 5 shows the distribution of labelling seen with the CMV-IE promoter at 7 days (plotted in red) against the distribution of labelling seen with the LAP at 21 days (plotted in blue). However, it must be appreciated that there is some variability between cases which probably reflects the difference in the severity of the infection seen in different animals.

In situ hybridization

To determine whether the latent behaviour of virus SC16-LβA reflects that of its wild-type (wt) parent, we infected animals with 2 × 10⁶ p.f.u. of wt SC16. Animals were sampled at 20 days (three animals) 40 days (one animal) and 1 year (one animal) p.i., and the brain and spinal cord were analysed by ISH for LATs. At all three time-points LAT labelling was seen in an identical distribution to the LacZ labelling seen at the same time-points in animals infected with virus SC16-LβA, indicating that SC16-LβA is a reliable marker of wt LAP activity in the CNS. Significantly, however, there were often at least tenfold more LAT-positive cells in the facial nucleus following wt virus infection than LacZ-positive cells following SC16-LβA infection. This is clear from Fig. 6, which shows LacZ expression in the locus coeruleus, hypoglossal and facial nuclei in animals infected with virus SC16-LβA, 1 month p.i., and the same brain regions expressing LATs in animals infected with wt virus (SC16).

As in animals infected with virus SC16-LβA, at 40 days p.i. the majority of the labelling was seen in motoneurones in the facial and hypoglossal nuclei. The occasional positive cell was also seen in the periaqueductal grey, the locus coeruleus and the reticular formation; in the spinal cord the majority of the LAT-positive neurones were motoneurones in the ventral horn. A few labelled cells were also seen in the central region (intermediomedial nucleus) and at the base of the dorsal horn. At the 1 year time-point, LAT-positive cells were predominantly seen in the facial nucleus, and an occasional motoneurone was labelled in the ventral spinal cord.
Fig. 4. For legend see facing page.
Finally, to confirm that the pattern of spread through the CNS during acute infection is the same for all the viruses utilized, we used a polyclonal rabbit anti-HSV antiserum to study the distribution of viral antigens in the brains of two animals infected with each virus at 7 days p.i. The same pattern of labelling was seen with all three viruses, indicating that they behave similarly in the way that they spread through the CNS (data not shown).

Discussion

The mouse ear model of infection (Hill et al., 1975) provides virus access to both the central and peripheral nervous systems. However, the spread of virus that occurs from the ear into the CNS after virus infection has not previously been described in detail. Lachmann & Elstathiou (1997) used this route of infection to study the expression of an IRES-linked reporter gene driven by the LAP in both the peripheral and central nervous systems. They detected LAP-driven reporter gene expression in both sensory ganglia and in the facial and hypoglossal nuclei between 42 and 300 days p.i. However, it was not clear from this study whether the facial and hypoglossal nuclei were the only sites in the CNS which were accessed by the virus following ear administration. It is possible that other CNS sites were infected, but that only the facial and hypoglossal nuclei supported long-term expression from the LAP. This suggestion would be consistent with previous work in the CNS which has suggested that not all neuronal subsets can support LAT expression (Kesari et al., 1996), and studies which have shown that many more sensory neurones contain viral DNA than are positive for LATs (Gressens & Martin, 1994; Ramakrishnan et al., 1994; Mehta et al., 1995; Maggioncalda et al., 1996).

In this paper we have addressed this issue by mapping the spread of HSV-1 from the ear through the spinal cord and brainstem, and comparing the sites of acute infection with those found to support transcriptionally active latency. The spread of virus was traced using SC16-C3b, which expresses the lacZ reporter gene under CMV-IE promoter control. The CMV-IE promoter is active in acutely infected cells, but is silenced during latency when the LAP is active. It is possible that there are areas in the CNS where latency is established without any prior expression from the CMV-IE promoter; however, since LAP activity can be detected in the same CNS regions which are labelled by CMV-IE promoter activity at early time-points, the CMV-IE promoter is probably an accurate marker of the acute phase distribution and spread of the virus. This result also shows that, as the virus spreads transneuronally, some of the infected cells in each brain region become lytically infected, whilst the rest establish latency, as has been observed by others using corneal routes of infection (Deatly et al., 1988; Stroop et al., 1984). On the basis of our cell counts it would appear that far more infected neurones establish latency than enter a lytic round of replication.

Cells labelled by the CMV-IE promoter were detected in the brainstem and spinal cord at 5 days p.i. The infection was restricted to a few sites only, suggesting that the infection had only just reached the CNS at this time-point. This finding is in agreement with previous studies (Kristensson et al., 1982; Stroop et al., 1984; LaVail et al., 1990; Margolis et al., 1989) which used corneal and snout routes of infection and in which the first signs of CNS infection were detected at 4 days p.i.

In the spinal cord at 5 days the infection was restricted to the dorsal horn and dorsal columns at the segmental level of entry of the virus. An interesting finding was that the virus had been transmitted to a column of cells in the dorsal horn (seen best in longitudinal section, Fig. 1), which corresponds to the sites of termination of sensory axon collaterals from the neurones innervating the ear pinna. This observation may help to explain the appearance of latently infected DRG cells at segmental levels above and below the level of the primary infection (Speck & Simmons, 1992, 1991; Lachmann et al., 1999). The dorsal horn is organized into continuous overlapping cell columns which receive inputs from DRG cells over the entire long axis of the spinal cord (for review see Noback & Demarest, 1984; Fitzgerald, 1994). Interneuronal transmission of the virus rostrally and caudally through the cell column would lead to the infection of sensory nerve terminals of DRG cells which enter (and terminate) at other segmental levels, permitting the establishment of latency at rostral and caudal ganglionic sites. HSV clearly passes trans-synaptically in the spinal cord since, by 7 days p.i., CMV-IE promoter-labelled motorneurones were seen bilaterally in the ventral horn. As there is no direct connection between spinal motorneurones and the mouse ear, the virus must have reached these cells by trans-synaptic transmission via spinal inter-neurones. In the brainstem a similar hypothesis has been suggested to account for the latent infection of neurones in all three divisions of the trigeminal ganglion following corneal inoculation with HSV-1 (Tullo et al., 1982).

Another way in which the virus might be spreading to ganglia at other segmental levels is by inter-axonal transmission in the dorsal columns. We frequently found that animals sampled after 7 days p.i. showed lytic damage to grey matter which corresponded to site of entry of the virus. The lack of viral antigen from the specific sites of ganglionic entry was consistent with the observation that HSV-1 does not spread through the CNS in a retrograde manner.
Fig. 5. For legend see facing page.
stretching across the entire dorsal column on the infected side. It is possible that axons from ganglionic levels further down the cord could have become infected en passage from adjacent infected axons and glia. However, there is some question over the ability of HSV to spread past the glial cells which surround each of the axons, as glia are believed to be relatively resistant to such infection (Card et al., 1993), although it is known that neuronal cell bodies lying adjacent to infected axons can themselves become infected (Ugolini et al., 1987).

The LAP was active in the spinal cord from 7 days p.i. Cells labelled by the LAP were found in the same anatomical sites as cells labelled by the CMV-IE promoter, and morphologically, appeared to be the same cell types. The only difference at the 7 day time-point was that the CMV-IE promoter labelled motoneurones in the ventral horn, and these were not found with the LAP until 15 days, consistent with a lag between infection, the establishment of latency, and activation of the LAP. By 21 days no cells were labelled by the CMV-IE promoter, but with the LAP, labelled cells were still present. These findings suggest that the LAP can function, at least until 21 days, in spinal cord interneurones and ventral horn motoneurones.

In the brainstem the CMV-IE promoter labelled cells at 5 days p.i., and showed the widest distribution of labelling at the 7 day time-point. The LAP was active in all of the same anatomical regions from 7 days p.i. In the facial nucleus, however, the acute infection involved only the medial part of the nucleus, yet latency was established in neurones distributed throughout the nucleus. The most likely explanation for this finding is that the virus had spread from lytically infected neuronal cell bodies in the medial part of the nucleus onto the dendrites of cells distributed throughout the nucleus, although an interaxonal model of spread in the facial nerve bundle (as described for the dorsal columns above) is also possible.

A significant finding was that by 34 days p.i. the majority of the labelled cells had disappeared and LAP-driven LacZ expression was only consistently detected in the 7 day time-point. The LAP was active in all of the same anatomical regions from 7 days p.i. In the facial nucleus, however, the acute infection involved only the medial part of the nucleus, yet latency was established in neurones distributed throughout the nucleus. The most likely explanation for this finding is that the virus had spread from lytically infected neuronal cell bodies in the medial part of the nucleus onto the dendrites of cells distributed throughout the nucleus, although an interaxonal model of spread in the facial nerve bundle (as described for the dorsal columns above) is also possible.

Fig. 5. In order to demonstrate that the LAP is active in the same anatomical sites in the CNS as those that are infected acutely, the distribution of LAP-LacZ (SC16-LβA) labelling seen in two animals at 21 days (shown by blue dots) is superimposed over the distribution of CMV-IE promoter-driven LacZ expression (SC16-C3b) seen in two animals at 7 days (shown by red dots). There is a close correspondence between regions of CMV-IE promoter labelling and regions of LAP-LacZ labelling. Significantly, panel 6 appears to show a large region of CMV-IE promoter labelling in the Pcr region which is not mirrored by the LAP. However, the majority of this CMV-IE labelling was in glial cells lying alongside the facial nerve and very few neurones in this region were infected; hence we would not expect to see long-term LAP activity in this region.

Another interesting finding in animals infected with wt SC16 was that we detected (by ISH) many (at least tenfold) more labelled cells in the facial nucleus than were β-Gal positive with SC16-LβA. It is possible that this disparity occurs because the immune system is removing some of the SC16-LβA-infected cells because they are expressing a foreign transgene, whilst the wt virus makes only LATs, which do not encode any antigen and hence escape immune surveillance. However, to determine the impact of the immune system in this study would require the use of transgenic mice tolerized to β-Gal. Another explanation for the detection of more LAP-labelled cells in the facial nucleus following wt virus infection might be that the wt virus has an intact LAT region and hence can establish latency in the CNS more efficiently than SC16-LβA. Previous studies have shown that LAT-negative mutants exhibit a reduced frequency of latency establishment in the trigeminal ganglion following corneal inoculation (Sawtell & Thompson, 1992; Thompson & Sawtell, 1997), but it is not known whether the LATs aid in the establishment of latency in CNS neurones in the same way. Indeed, it has been shown that the deletion of LATs does not affect latency establishment in lumbrosacral ganglia following footpad infection (Sawtell & Thompson, 1992), so the role of LATs in the establishment of CNS latency cannot be predicted.

The genome copy number in the latently infected neurone may also play a role in the duration of expression through a template effect. It is possible that the facial nucleus, being a first order site of infection, might have a greater copy number than other subsequently infected sites within the CNS, producing an apparent preferential persistence of expression in this site. To test this hypothesis would require the delivery of known amounts of virus particles to defined cell populations within the CNS, which is best done by stereotaxic injection of a replication-defective virus. We are currently carrying out such experiments to determine whether virus input has a significant consequence for long-term expression in the CNS.

Finally, it has previously been shown that LAP-driven β-Gal expression resulted in tenfold more cells positive for β-Gal transcript than cells positive for β-Gal enzyme, suggesting that sequences at the 5' end of the β-Gal transcript influence translation of the β-Gal message (Huang et al., 1997). Although the lacZ gene used in the current study is preceded by an IRES element to circumvent such translational difficulties, further
Fig. 6. A comparison of LAP-LacZ (SC16-LβA) and LAP-LAT (wt SC16) expression in the facial nucleus (A, B), locus coeruleus (C, D) and hypoglossal nuclei (E, F) at 21 days p.i. Labelled cells are seen in each of these sites with both viruses, demonstrating that SC16-LβA (in which the reporter gene lacZ is under the control of the LAP) is an accurate indicator of wt LAP activity in the CNS.
In conclusion, we have shown that following ear inoculation with HSV-1 the virus spreads to a reproducible series of sites in the CNS. By using a virus (SC16-LP3) in which lacZ is driven by the LAP we have detected β-Gal expression in all of the same sites in the CNS that were accessed by the virus acutely and also in which LATs were detected by ISH following wt virus infection. These data demonstrate that the LAP can function for at least 21 days in many, if not all, CNS neuronal cell types in the brainstem and spinal cord. With the passage of time, however, there was a reduction in the distribution of labelling, and LAP-driven reporter gene expression was detected predominantly in the facial nucleus and sometimes also in the hypoglossal nucleus and spinal motorneurones. A similar result was obtained following wt virus infection, in which LAP activity was detected by ISH for LATs. These findings suggest that the preferential long-term activity of the LAP in motorneurones is probably not a consequence of immune clearance but instead that the LAP may function selectively, or at least more efficiently in some CNS neurones than others.

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References


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