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An analysis of herpes simplex virus gene expression during latency establishment and reactivation

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In order to facilitate an analysis of the pattern of herpes simplex virus gene expression during latency establishment and reactivation, recombinant viruses containing the *lacZ* reporter gene under control of either the immediate early 110 (IE110) promoter or the latency-associated promoter have been constructed. Histochemical staining of ganglia taken from mice infected with these viruses allows for the rapid identification and quantification of sensory neurones in which these two promoters are active. Using the mouse ear model, this study demonstrates that, during the establishment of latency *in vivo*, IE110 promoter activity is only detectable in ganglia which provide innervation to the site of virus inoculation. Latency, however, is efficiently established not only in these ganglia, but also in adjacent ganglia whose neurones do not innervate the ear, and in which there was no evidence of IE110 expression during the acute phase of infection. This implies that replication-competent virus can efficiently establish latency in the absence of detectable IE110 expression. In addition, it has been possible to investigate viral gene expression in neurones following ganglionic explant culture by monitoring IE110 promoter-driven *lacZ* expression within reactivating neurones. This study shows that virus can be reactivated from all latently infected ganglia, but that reactivation appears to be more efficient from ganglia which provide innervation to the site of infection. The implications of these results for the mechanisms involved in latency establishment and reactivation are discussed.

Introduction

Lytic cycle herpes simplex virus type 1 (HSV-1) gene expression has been extensively studied in non-neuronal tissue culture cells (Wagner *et al.*, 1995). Transcription of the viral immediate early (IE) genes is activated by the binding of the virion transactivator protein, VP16, complexed with the host transcription factors Oct-1 and HCF, to a shared sequence motif present in all five IE gene promoters (Campbell *et al.*, 1984; Kristie & Sharp, 1993; O'Hare *et al.*, 1988). IE gene products have regulatory effects on viral gene transcription, and are responsible for the co-ordinate activation of viral early and late gene expression (Preston, 1979; Purves *et al.*, 1993; Sacks *et al.*, 1985; Stow & Stow, 1986). Lytic cycle gene expression is, therefore, dependent on the successful activation of IE transcription.

HSV establishes a latent infection within sensory neurones that innervate the site of primary infection. During latency, all the lytic cycle promoters of the virus are silenced, and only a single transcription unit, encoding the latency-associated transcripts (LATs), remains active (Stevens *et al.*, 1987). The function of the LATs is not fully understood (see Wagner & Bloom, 1997). They are not essential for any phase of the virus life-cycle, but they have been reported to function both in the establishment of latency (Thompson & Sawtell, 1997) and in reactivation (Bloom *et al.*, 1996; Hill *et al.*, 1997; Perng *et al.*, 1996).

A number of viruses containing mutations within the virion transactivator protein, VP16 (Ace *et al.*, 1989), and/or one or more of the IE genes (DeLuca *et al.*, 1985; Preston *et al.*, 1997; Wu *et al.*, 1996) have been described. Such viruses are unable to initiate lytic cycle infection in non-complementing cells in culture (Ace *et al.*, 1989; DeLuca *et al.*, 1985; Jamieson *et al.*, 1995; Preston *et al.*, 1997; Wu *et al.*, 1996). However, a number of such mutants can efficiently establish latent infection in neurones *in vivo* (Chiocca *et al.*, 1990; Ecob-Prince *et al.*, 1993; Sedarati *et al.*, 1993). These data suggest that latency can be established in the absence of any lytic cycle gene expression, and may therefore represent a 'default' pathway which occurs

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following virus entry into a cell that is non-permissive for the initiation of lytic cycle gene expression.

Such a default model of latency is consistent with studies of HSV latency using well characterized mouse models. Using the zosteriform model of infection, Speck & Simmons (1991) have shown that, during latency, LAT-expressing neurones can be found in sensory ganglia in which there had been no detectable lytic cycle gene expression during the establishment phase of infection. Furthermore, during acute infection of ganglia innervating the site of inoculation, two separate populations of neurones have been identified, one expressing lytic cycle genes and the other expressing only the LATs (Margolis *et al*., 1992; Speck & Simmons, 1992), suggesting that the lytic and latent pathways of gene expression diverge from each other immediately upon infection of the neurone. In cultured sensory neurones, however, it has been reported that expression of one of the IE genes, IE110, is required for the efficient establishment of latency (Wilcox *et al*., 1997). This might suggest that the branch point between lytic and latent pathways of infection may in fact occur after the initiation of IE gene expression.

Previous studies have used immunohistochemical detection of viral antigen as a marker for cells undergoing lytic cycle gene expression *in vivo* (Margolis *et al*., 1992; Speck & Simmons, 1991, 1992). However, this technique may not allow the detection of neurones in which a low level of IE gene expression precedes the establishment of latency. In the work described herein, we have utilized recombinant viruses in which a sensitive reporter gene, *lacZ*, is placed under control of either the IE110 promoter or the latency-associated promoter (LAP) to address the question of whether activation of IE gene expression necessarily precedes the establishment of latency *in vivo* and to look at early events following explant reactivation.

Methods

■ **Cells and viruses.** Viruses were propagated and assayed on Vero cells (Lachmann & Efstathiou, 1997). All viruses are derived from HSV-1 strain SC16 (Hill *et al*., 1975).

■ **Plasmids.** pHD5 is a plasmid designed to allow recombination of foreign sequences into the US5 gene of HSV-1 strain SC16 (Balan *et al*., 1994).

pPO48 was a gift from P. O'Hare (Marie Curie Research Institute, UK). It contains a 968 bp *Sac*I/*Nco*I fragment, derived from HSV-1 strain KOS (nucleotides 1294–2262), comprising the IE110 promoter and extending from position −818 to position +150 with respect to the transcription start site (O'Hare & Goding, 1988), flanked by a *Hin*dIII site at its 3′ end and a *Sal*I site at its 5′ end.

To make plasmid pIE110lacZ, the *lacZ* gene and simian virus 40 polyadenylation signal (SV40pA) were excised from pNASS*β* (Clontech Laboratories) as a 3·9 kb *Xho*I/*Sal*I fragment and cloned into the *Sal*I site of pPO48, such that transcription of *lacZ* was driven by the IE110 promoter.

The plasmid pHD5IE110 was made by blunt end cloning of the *Hin*dIII/*Sal*I cassette from pIE110lacZ into *Eco*RV-cut pHD5, such that transcription from the IE110 promoter was in the same direction as the US5 gene.

■ **Construction of recombinant virus SC16 110lacZ.** pHD5IE110 was linearized by *Sca*I digestion and 3 μg of this plasmid DNA was cotransfected into Vero cells with 10 μg of high molecular mass SC16-infected cell DNA by a $CaCl_2$/DMSO boost method (Stow & Wilkie, 1976). Recombinant, *β*-Gal-positive plaques were selected by successive rounds of plaque picking under an agarose overlay as described previously (Lachmann & Efstathiou, 1997).

■ **Mouse infections.** Groups of 5–6-week-old female BALB/c mice (Harlan) were infected with 2×10^6 p.f.u. virus in a volume of 20 μl PBS by subcutaneous injection into the pinna of the left ear. Upon sampling, left dorsal root ganglia (DRG) C2 to C6 were dissected and either processed immediately for histochemical detection of *β*-Gal activity or *in situ* hybridization (ISH), or placed in 0·5 ml Glasgow Modified Eagle's Medium with 10% foetal calf serum in a microcentrifuge tube for explant reactivation. Ganglia for explant reactivation were placed in a 37 °C gassed 5% CO_2 incubator. On removal from the incubator, explanted ganglia were fixed and stained for *β*-Gal activity.

■ **Histochemical *β*-Gal detection.** *β*-Gal-positive neurones were detected by histochemical staining of whole ganglia with X-Gal, as described previously (Lachmann & Efstathiou, 1997). Ganglia were stained for 3 h prior to clarification in glycerol and enumeration of positive neurones.

■ ***In situ* hybridization.** ISH was performed as previously described using major LAT-specific digoxigenin-labelled probes made by transcription of plasmid pSLAT2 (Arthur *et al*., 1993; Lachmann & Efstathiou, 1997).

Results

Construction of recombinant viruses

We have previously described the construction of virus SC16 L*β*A (Lachmann & Efstathiou, 1997). This virus has a cassette containing the *lacZ* gene linked to the encephalomyocarditis virus (EMCV) IRES inserted into the LAT region approximately 1·5 kb downstream of the primary LAT (minor LAT or mLAT) transcription start site (Fig. 1*a*). Elements required for long-term activity of the LAP within latently infected neurones have been maintained intact in this recombinant virus, which is able to express *β*-Gal during neuronal latency (Lachmann & Efstathiou, 1997).

Virus SC16 110lacZ contains a 968 bp promoter fragment which extends from position −818 to position +150 with respect to the IE110 transcription start site (O'Hare & Goding, 1988) linked to *lacZ* and inserted into the non-essential US5 locus of HSV-1 strain SC16 (Balan *et al*., 1994) (Fig. 1*a*).

When these viruses are propagated in tissue culture, the SC16 110lacZ virus produces 'blue', *β*-Gal-positive plaques (Fig. 1*b*), confirming that the IE110 promoter contained within the insertion cassette is active during lytic infection. In contrast, SC16 L*β*A produces a characteristic 'speckled' plaque phenotype with *β*-Gal expression only detectable in a proportion of the infected cells (Fig. 1*c*).

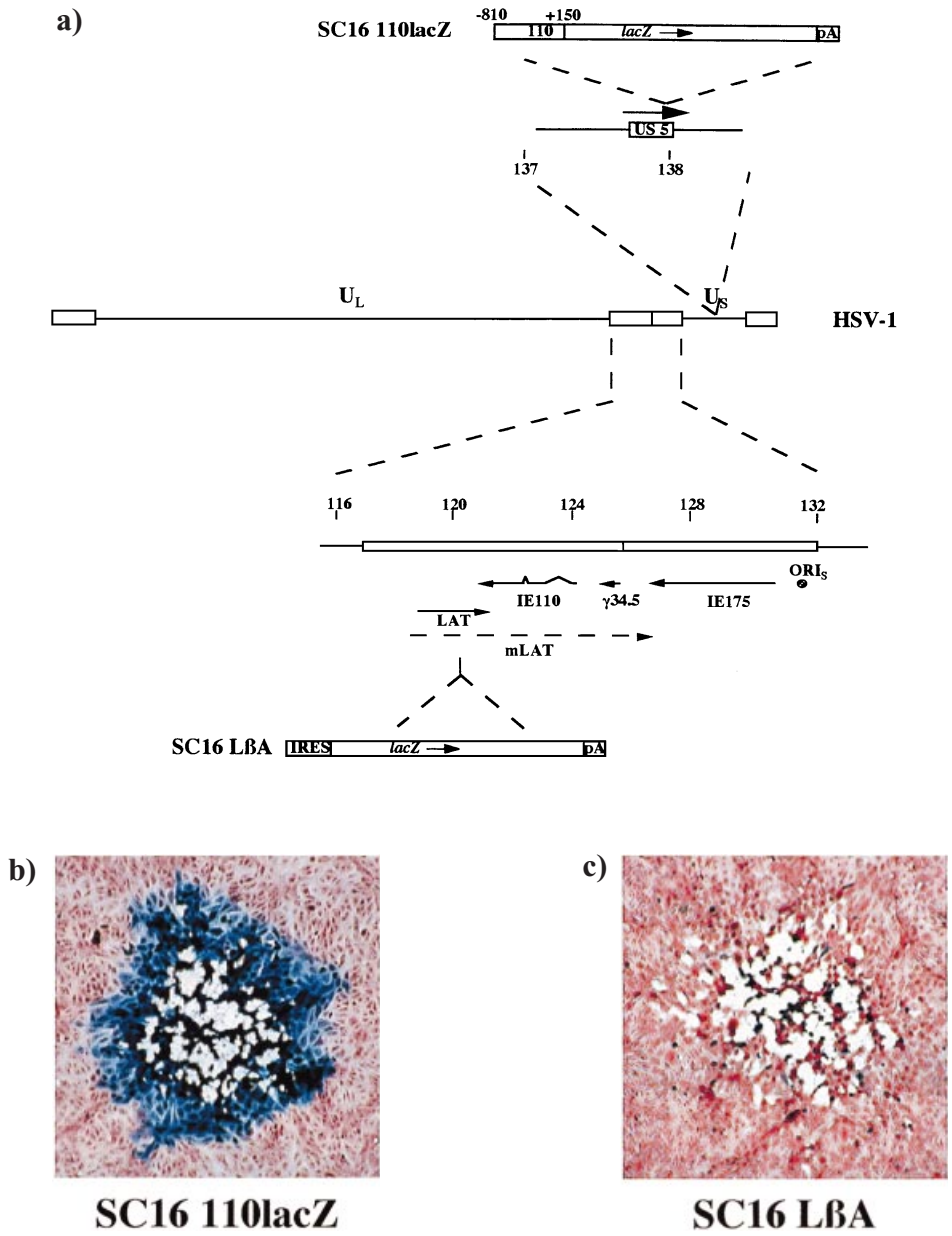

Fig. 1. Structure of recombinant viruses and their plaque morphology. (*a*) A schematic diagram of the HSV-1 genome is shown with unique long (U_L) and unique short (U_S) regions each flanked by inverted terminal repeats. The upper expansion shows the US5 (gJ) gene and the insertion locus used in construction of the virus SC16 110lacZ. Above, the IE110 promoter–*lacZ*–SV40 pA cassette present in SC16 110lacZ is shown. The lower expansion shows the region of the terminal repeats (which is diploid in the circular latent genome). The positions of the lytic cycle IE110, γ34.5 and IE175 transcripts are indicated, as well as those of the primary latency-associated transcript (mLAT) and the 2 kb major LAT intron. In the recombinant virus SC16 L*β*A (shown below) a cassette consisting of the EMCV IRES linked to *lacZ* with the CMV pA signal has been inserted into the major LAT-coding sequence. Viral structures were confirmed by Southern hybridization (data not shown). (*b*) A 2 day SC16 110lacZ plaque on a *β*-Gal-stained Vero cell monolayer. All infected cells are strongly positive for *β*-Gal. (*c*) A 2 day SC16 L*β*A plaque on a *β*-Gal-stained Vero cell monolayer. Macroscopically these plaques appear to be 'white', and *β*-Gal-negative. Under the microscope, however, the plaques have a 'speckled' appearance, and contain some *β*-Gal-positive cells in their centres.

Characterization of transcription from the IE110 promoter and the LAP during the establishment of latency

In order to investigate gene expression from the IE110 promoter and the LAP during the establishment of latency in the murine peripheral nervous system, groups of mice were infected with 2×10^6 p.f.u. of either SC16 110lacZ or SC16 L*β*A by subcutaneous injection into the pinna of the left ear (Hill *et al.*, 1975). At 2, 4, 6 and 31 days after infection, five mice from each group were sampled, and five cervical DRG, C2 to

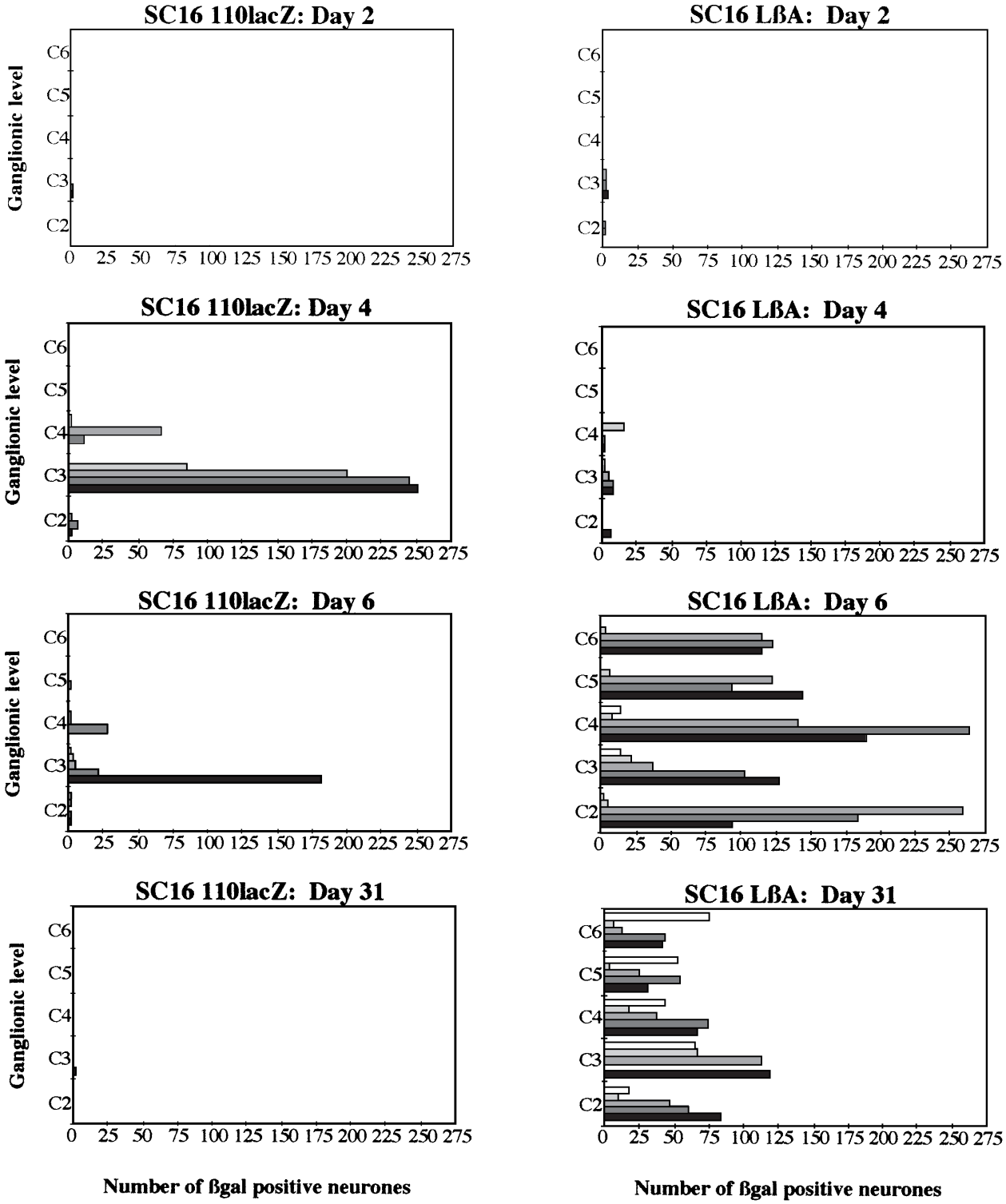

Fig. 2. β-Gal expression in DRG during the establishment of latency by SC16 110lacZ and SC16 LβA. Bar graphs showing the number of β-Gal-positive neurones counted in each ganglion at 2, 4, 6 and 31 days after infection with either SC16 110lacZ or SC16 LβA. Ganglia were stained with X-Gal for 3 h prior to clarification in glycerol. Ganglia were whole-mounted under coverslips, and β-Gal-positive neurones were counted under the microscope. Each graph represents the data from five animals (A to E). For each animal, the number of neurones counted in each of the DRG C2 to C6 is shown as a shaded bar, with different shading being used to represent different mice.

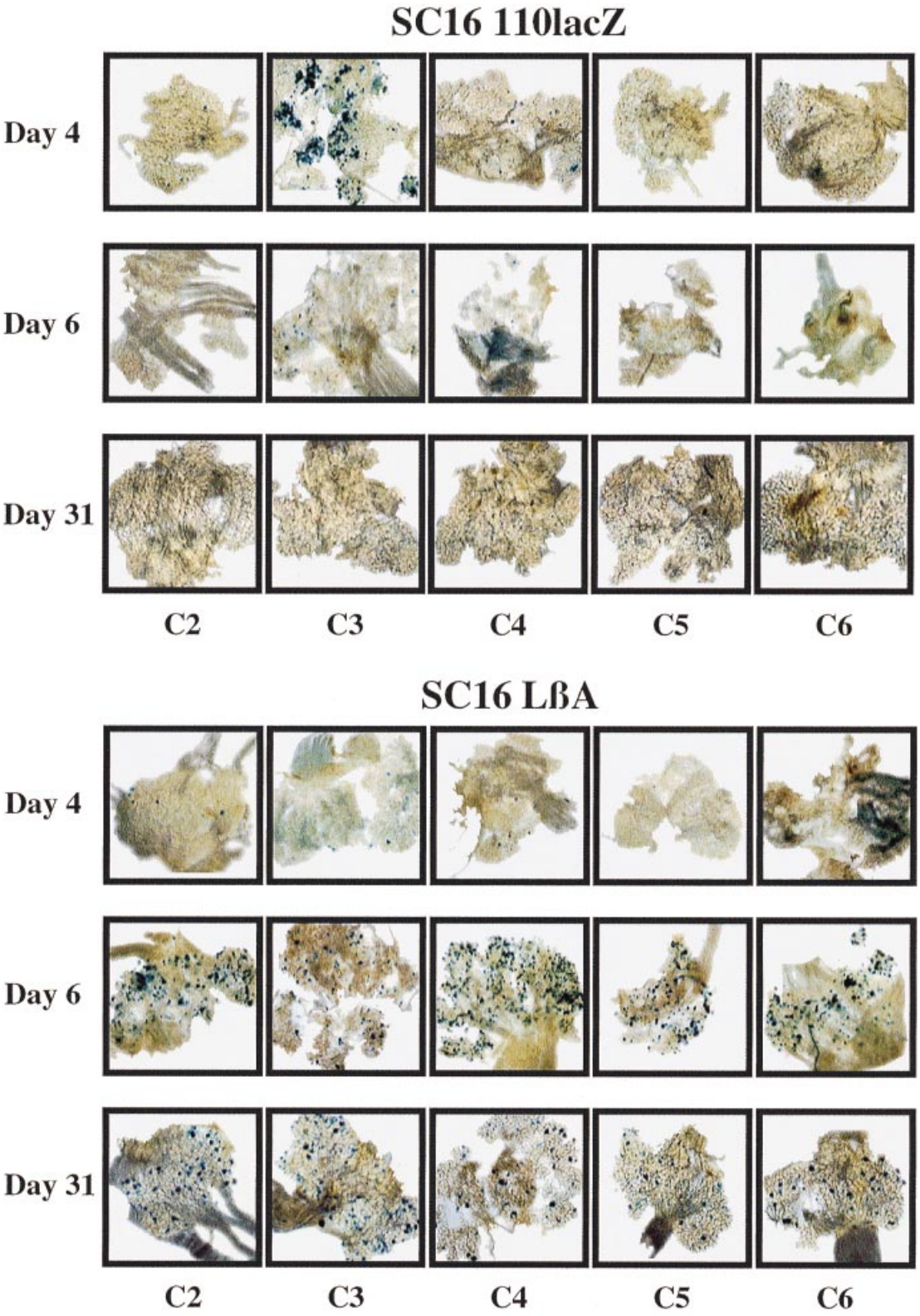

Fig. 3. Photomicrographs of β-Gal-stained DRG from mice infected with SC16 110lacZ and SC16 LβA. Whole-mounted DRG C2 to C6 are shown from a representative mouse from each experimental group sacrificed at 4, 6 and 31 days post-infection. Ganglia have been histochemically stained for 3 h using X-Gal, then clarified in glycerol.

C6, were dissected and individually stained for β-Gal activity using X-Gal. By selecting these five ganglionic levels, we were able to assess gene expression in ganglia (C2 to C4) that provided innervation to the head and neck, including the site of inoculation, and in ganglia whose dermatomes lie in the upper limb (C5 and C6) and which contain no neurones supplying sensation to the ear. The numbers of β-Gal-positive neurones present in each ganglion are shown graphically in Fig. 2, and representative photomicrographs of individual ganglia are shown in Fig. 3.

The left ears, containing the site of inoculation, were also dissected from the mice sacrificed at day 4 post-infection. The individual ears were homogenized and assayed for virus infectivity. The average ear titres obtained were 5·1 $\log_{10}$ p.f.u. (SE 4·5 $\log_{10}$ p.f.u.) from the SC16 LβA-infected mice and 4·7 $\log_{10}$ p.f.u. (SE 3·8 $\log_{10}$ p.f.u.) from the SC16 110lacZ-infected mice, indicating that both viruses replicated with comparable efficiencies at the periphery.

At day 2 post-infection, very few neurones from either experimental group were β-Gal-positive. Only two neurones were positive in one of five mice infected with SC16 110lacZ, all at the C3 level. Eight positive neurones were seen in three of five mice from the SC16 LβA-infected group, and these were spread between the C3 and C2 levels (Fig. 2).

By day 4 post-infection, an average of 156 β-Gal-positive neurones per ganglion were detected at the C3 level in four of five mice infected with SC16 110lacZ. Fewer positive neurones were detected at the adjoining C2 and C4 levels, which supply innervation to the head and neck, and no β-Gal-positive neurones were seen at the C5 and C6 levels, which innervate the upper limb. The staining seen was strong and relatively uniform from cell to cell (Fig. 3). In the mice infected with SC16 LβA there were only a few neurones with detectable levels of LAP-driven *lacZ* expression. As in the SC16 110lacZ group, positive neurones were only seen in ganglia C2 to C4, but the intensity of staining seen was variable, and, on the whole, less than that which was observed when *lacZ* expression was driven by the IE110 promoter (Fig. 3).

At day 6 post-infection, the numbers of neurones positive for IE110 promoter-driven *lacZ* expression had decreased markedly from day 4 post-infection. In three of the five animals infected with SC16 110lacZ there were less than ten β-Gal-positive neurones remaining per mouse. In the fourth mouse there were a total of 51 neurones, and this mouse was the only one infected with the SC16 110lacZ virus in which β-Gal-positive neurones were detected outside the C2 to C4 levels (two positive neurones were detected in the C5 ganglion). The fifth mouse showed a pattern of *lacZ* expression similar to that seen in the mice sacrificed at day 4 post-infection, and it seems likely that the peak of lytic cycle infection seen in this animal was lagging behind those seen in the majority of animals in the group.

In contrast to the results obtained at the day 4 time-point, at day 6 post-infection, the animals infected with SC16 LβA demonstrated much higher levels of β-Gal expression than those infected with SC16 110lacZ sampled at the same time-point (Fig. 2). The mean numbers of positive neurones per ganglion were 108 at C2, 60 at C3, 123 at C4, 73 at C5 and 88 at C6. Thus, LAP-driven *lacZ* expression was detected at the C5 and C6 levels, in which it had not been possible to detect significant IE110 promoter-driven *lacZ* expression.

The day 31 time-point was taken to represent fully established latent infection. As expected, there was essentially no detectable IE110 promoter activity in mice latently infected with SC16 110lacZ. Only a single, faintly staining, β-Gal-positive neurone was detected in the 25 ganglia which were examined. In contrast, β-Gal-positive neurones continued to be observed at all ganglionic levels sampled in the mice latently infected with SC16 LβA. Overall, the numbers of β-Gal-positive neurones seen were fewer than at day 6 post-infection, and the distribution of β-Gal-positive neurones had changed, but, because of the mouse to mouse variation in levels of latency established, the significance of these observations is unclear. The highest numbers of positive neurones were now being consistently found at the C3 level, where the average number of β-Gal-positive neurones per ganglion had actually risen from 60 at day 6 to 90 at day 31 post-infection. LAP-driven *lacZ* expression could still be detected at the C5 and C6 levels in an average 33 and 35 positive neurones per ganglion respectively.

Therefore, the results obtained with SC16 LβA suggest that, after ear infection, HSV is able to establish transcriptionally active latency at all ganglionic levels from C2 to C6. In order to confirm that SC16 110lacZ was also capable of efficiently establishing latency at the C5 and C6 levels, more mice from the SC16 110lacZ-infected group were sampled at 31 days after infection to allow the level of latency established to be assessed by ISH detection of LATs. DRG C2 to C4 from six mice were pooled, as were DRG C5 and C6. ISH analysis of pooled C2 to C4 ganglia detected 744 LAT-positive neuronal profiles in 79 ganglionic sections examined – an average of 9·4 LAT-positive neuronal profiles per ganglionic section. ISH analysis of pooled C5 and C6 ganglia detected 161 LAT-positive neuronal profiles in 19 ganglionic sections examined – an average of 8·5 positive neuronal profiles per ganglionic section. These data show that this virus was able to establish transcriptionally active latency within the C5 and C6 ganglia with a similar efficiency to that established at the C2 to C4 levels.

Therefore, although during the establishment of latency by SC16 110lacZ transcription from the IE110 promoter was only detected in the ganglia supplying innervation to the site of inoculation, latency was also efficiently established in adjacent ganglia. This implies that, in at least a proportion of latently infected neurones, there is no detectable IE gene expression preceding the establishment of latency, indicating that a fully replication-competent virus can establish default latency.

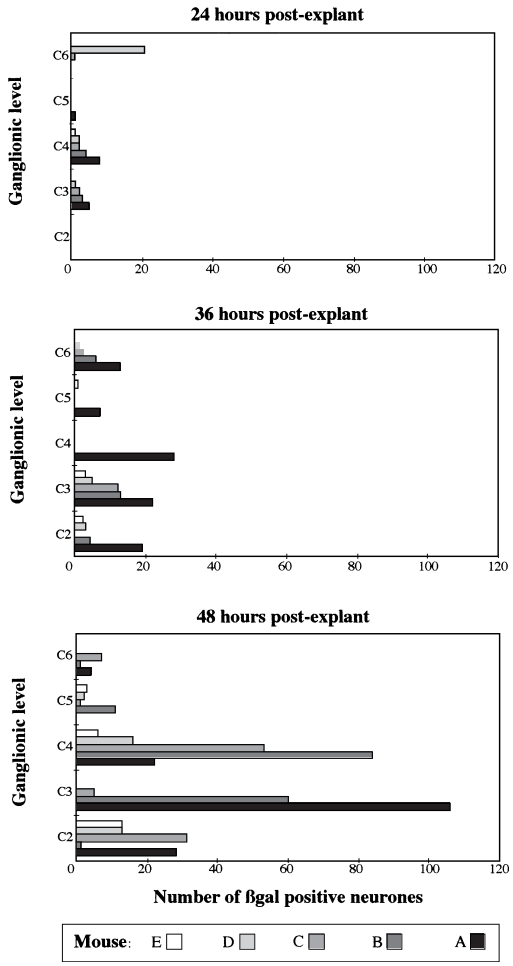

Fig. 4. β-Gal expression in DRG during explant reactivation of SC16 110lacZ. Bar graphs showing the number of β-Gal-positive neurones counted in ganglia latently infected with SC16 110lacZ and explanted for 24, 36 or 48 h prior to histochemical staining. Ganglia were stained with X-Gal for 3 h prior to clarification in glycerol. Ganglia were whole-mounted under coverslips, and β-Gal-positive neurones counted under the microscope. Each graph represents the data from five animals (A to E). For each animal, the number of neurones counted in each of the DRG C2 to C6 is shown as a shaded bar, with different shading being used to represent different mice.

The assessment of IE110 promoter activity during reactivation of SC16 110lacZ

The ability of HSV to establish latency in sensory neurones that do not directly innervate the site of peripheral infection is also observed following corneal inoculation, when latently infected cells can be found within the maxillary and mandibular as well as the ophthalmic divisions of the trigeminal ganglion (Tullo *et al.*, 1982). This is also likely to be the situation after natural oropharyngeal infection in man. Yet the vast majority of recurrences occur in a single division of the nerve, corresponding to the site of primary infection. Slobedman *et al.* (1994) have shown that, in the zosteriform model of mouse infection, the average number of HSV genomes present per major LAT-expressing cell is about 200 in the ganglia which innervate the site of inoculation, but only about 20 in the adjacent ganglia which supply neighbouring dermatomes. Sawtell and colleagues have recently reported that the efficiency of hyperthermia-induced reactivation from trigeminal ganglia correlates both with the number of latently infected neurones present (Sawtell, 1998) and with the copy number of viral DNA within latently infected neurones (Sawtell *et al.*, 1998). Thus, differences in the copy number of virus latency could provide an explanation for the differences in reactivation efficiency between the primary latently infected neurones (those which directly innervate the site of primary infection) and the secondary, distal, latently infected neurones.

Production of functional IE110 has been shown to be important in reactivation of HSV both *in vivo* (Leib *et al.*, 1989) and in an *in vitro* model of latency (Harris & Preston, 1991). Thus, detection of IE110 promoter-driven *lacZ* expression would be predicted to provide a sensitive marker for virus reactivation.

In order to investigate IE110 promoter activity during explant reactivation, a further group of mice were infected with SC16 110lacZ. At day 26 post-infection, 15 animals were sacrificed and DRG C2 to C6 were dissected from each animal. Each individual ganglion was placed in medium in a separate tube and transferred to a 37 °C incubator. At 24, 36 and 48 h post-explantation, the ganglia from five mice were removed from the incubator and histochemically stained for β-Gal activity. The number of β-Gal-positive neurones in each ganglion was counted, and the results are shown in Fig. 4. Photomicrographs of ganglia, from representative mice, which were stained 36 and 48 h post-explantation, are shown in Fig. 5.

At 24 h post-explantation, there were β-Gal-positive neurones present in all five animals sampled (Fig. 4). The majority of positive neurones were seen at the C3 and C4 ganglia. In all animals, the staining intensity was weak compared to that seen at the later time-points (data not shown). By 36 h post-explantation, an increase in the number of β-Gal-positive neurones was observed, and the staining intensity had increased (Figs 4 and 5). All five of the C3 ganglia, and four out of the five C2 ganglia sampled contained β-Gal-positive neurones. At the C4 to C6 levels, only five out of 15 ganglia contained positive neurones, three of which were from a single animal.

At 48 h post-explantation, 20 out of the 25 ganglia sampled contained β-Gal-positive neurones. The intensity of staining observed was greater than at the earlier time-points, and there was some evidence of secondary spread of virus to form small foci of infection around reactivating neurones (e.g. the C3 ganglion shown in Fig. 5). Where it was not possible to resolve individual neurones, such foci were counted as a single reactivating cell. The average number of neurones seen at each level was 17 at C2, 34 at C3, 36 at C4, three at C5 and two at C6. Thus, there was an approximately tenfold difference between the numbers of neurones positive for IE110 promoter-

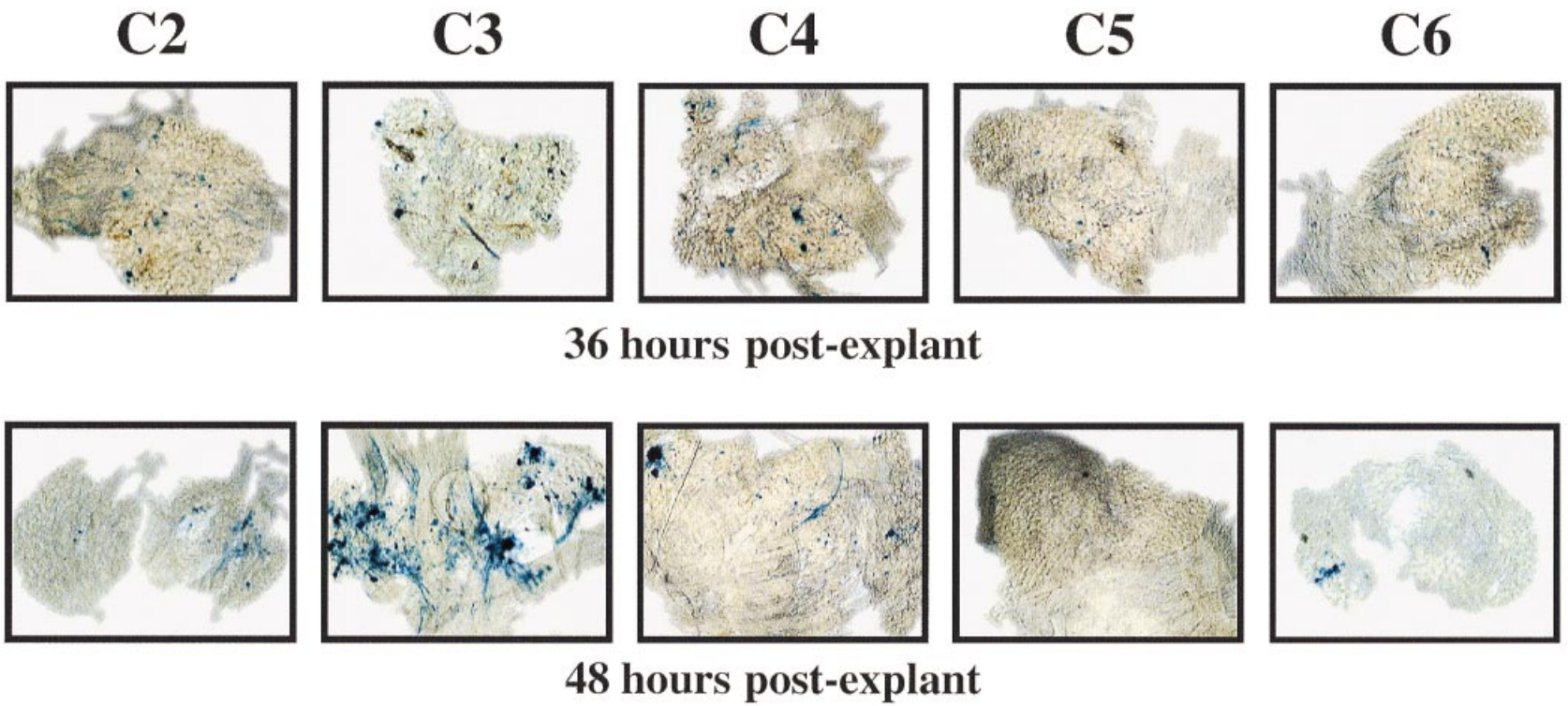

Fig. 5. Photomicrographs of explanted DRG histochemically stained for β-Gal. Whole-mounted DRG C2 to C6 are shown from representative mice, latently infected with SC16 110lacZ and explanted for 36 or 48 h prior to histochemical staining. Ganglia were stained for 3 h using X-Gal, then clarified in glycerol.

driven β-Gal expression at the C3 and C4 levels, which provide innervation to the ear, and in which IE110 gene expression could be detected in the acute phase of infection, and the number of β-Gal-positive neurones seen at the C5 and C6 levels, which provide no nerve supply to the ear, and in which no IE110 gene expression was detected at early time-points after infection.

In order to assess the levels of ganglionic latency which had been established in this group of experimental animals, a further seven mice were sacrificed at day 45 post-infection, and DRG C2 to C4 were dissected and pooled, separately from C5 and C6. Both sets of pooled ganglia were processed for ISH, which was performed with a major LAT-specific probe. In the C2 to C4 ganglia 924 LAT-positive neuronal profiles were detected in 186 ganglionic sections examined – an average of 5·0 LAT-positive neuronal profiles per ganglionic section. In the C5 and C6 ganglia the level of latency established was somewhat lower, with 292 LAT-positive neuronal profiles detected in 110 ganglionic sections examined – an average of 2·7 LAT-positive neuronal profiles per ganglionic section.

Therefore, as assessed by ISH, the level of latency established in the C2 to C4 ganglia from the SC16 110lacZ-infected animals was approximately twice that established at the C5 and C6 ganglia (which would be in keeping with the results obtained when ganglia latently infected with SC16 LβA are stained for β-Gal; Fig. 2). The tenfold difference seen between these groups of ganglia in the numbers of neurones reactivating after explantation may therefore represent a genuine functional difference between latency established at these different ganglionic levels.

Discussion

In the first set of experiments described here we set out to investigate patterns of viral gene expression during the establishment of latency by HSV-1. Two recombinant viruses, one in which *lacZ* expression is driven by the IE110 promoter (SC16 110lacZ) and one in which it is driven by the LAP (SC16 LβA), were constructed and used to monitor viral gene expression in cervical DRG during the establishment of latency following ear inoculation.

The numbers of neurones expressing lytic cycle genes after infection with SC16 110lacZ, as assessed by IE110 promoter-driven *lacZ* expression, peaked between 2 and 4 days post-infection, and then rapidly declined. IE110 promoter-driven gene expression was confined to those ganglionic levels that provided sensory innervation to the site of inoculation on the ear (primarily C3), and was not seen in adjacent ganglia which innervate only the upper limb (C5 and C6).

Latent cycle gene expression was detected by histochemical staining of ganglia from mice infected with SC16 LβA. Significant numbers of neurones transcribing *lacZ* from the LAP were not detected until 6 days post-infection, when they appeared synchronously at all ganglionic levels studied, not just those innervating the site of inoculation. Neurones harbouring transcriptionally active latent genomes continued to be observed at 1 month post-infection, and our previous experiments indicate that the numbers of neurones expressing β-Gal from the LAP remains stable over many months in mice latently infected with SC16 LβA (Lachmann & Efstathiou, 1997). In order to confirm that virus SC16 110lacZ was behaving in a similar manner, we performed ISH for LAT on pooled ganglia taken from latently infected mice. The results from two separate experiments confirm that this virus is able to establish latency at the C5 and C6 levels. The numbers of latently infected neurones present in the C2 to C4 ganglia, as assessed by ISH, were approximately twice those present in the C5 and C6 ganglia, a result which is consistent with that obtained when β-Gal-expressing neurones are counted in ganglia latently infected with SC16 LβA (Fig. 2).

The plaque phenotype of SC16 110lacZ in tissue culture indicates that abundant β-Gal activity is detectable in all lytically infected cells. It is possible, however, that the sensitivity of β-Gal detection in fixed DRG is not sufficient to detect very low levels of *lacZ* expression from the IE110 promoter. Whether or not there is a threshold below which we have not been able to detect IE110 promoter activity, the qualitative differences in β-Gal staining seen at the different ganglionic levels during establishment of latency with SC16 110lacZ are striking.

These data demonstrate that a fully replication-competent HSV is able to establish latency in DRG where there has been no detectable IE gene expression, and confirms that the default model of latency is relevant to wild-type viruses as well as replication-defective mutants. Speck & Simmons (1991) have previously reported similar results using the zosteriform model of infection, where C57BL mice (a relatively resistant strain) are inoculated with a wild-type virus by scarification of the flank. In their experiments, lytic cycle gene expression was assessed by histochemical detection of viral antigens in ganglionic sections and levels of latency were measured by ISH for LAT and the ability to reactivate virus. Our approach has been to use reporter gene expression from the IE110 promoter or LAP to allow the sensitive identification of neurones transcribing from these promoters within individual whole-mounted ganglia.

Using the ear route of infection, the restriction of productive cycle gene expression to neurones directly innervating the site of peripheral inoculation is striking. Following a first round of replication within sensory neurones innervating the site of inoculation, virus is able to gain access to adjacent DRG, but here the pattern of viral gene expression seen is quite different, with no detectable initiation of transcription from the IE110 promoter and the immediate establishment of a latent pattern of gene expression. Five days after inoculation of the ear, viral gene expression can be detected within the upper cervical spinal cord in the dorsal root entry zones (C. Smith, R. H. Lachmann & S. Efstathiou, unpublished data). Axonal branches of sensory neurones from several adjacent DRG

synapse with interneurones within this region of the cord and it seems likely that virus replication at this site allows virus access to the C5 and C6 ganglia, which do not directly innervate the ear.

Why should these subsequently infected sensory neurones be non-permissive for virus replication and instead favour the establishment of latency? It is possible that the restriction of viral gene expression is immune-mediated. Alternatively, the initiation of IE gene expression may be determined by which route the virus enters the neurone. Neurones may be permissive for IE gene expression if the virus enters the cell body by transport from the epithelial surface up the peripheral branch of the axon, but non-permissive if virus entry is from the CNS via the central branch of the axon. A third possibility is that sensory neurones are in general non-permissive for lytic viral gene expression, and hence favour the establishment of latency by default. This model requires there to be a physiological change in neurones which innervate the site of infection, rendering them permissive for initiation of the productive cycle. It has been shown that scarification of the cornea alone can result in the induction of the transcription factors c-Jun and Oct-1 within sensory neurones of the trigeminal ganglion (Valyi Nagy *et al*., 1991). Hence, virus replication or trauma at an epithelial surface can lead to changes in the transcriptional milieu of the innervating neurones which could act to 'prime' them and allow the initiation of IE gene expression. Such a mechanism would allow the virus to undergo a single round of replication in the relevant DRG, resulting in the production of progeny virus and seeding to other, remote sites in the nervous system. The second order sensory neurones remain 'unprimed', and are non-permissive for IE gene expression, leading to the establishment of default latency. In this way, the virus is able to spread within the nervous system, and establish a reservoir of latently infected neurones at different sites, whilst limiting productive infection to a small population of neurones, thus minimizing the risk of uncontrolled replication within the nervous system.

Using IE110 mutant viruses to infect cultured sensory neurones, Wilcox *et al*. (1997) found that functional IE110 was required for efficient latency establishment. Using a fully replication-competent virus *in vivo*, we have found no evidence of IE110 transcription during the establishment of latency in DRG that do not innervate the site of inoculation. Infection of neurones in culture, however, will resemble events occurring in the ganglia which directly innervate the site of inoculation, and in which we were able to detect abundant IE110 promoter activity. It is therefore possible that there is an IE110 requirement for the efficient establishment of latency in this population of neurones.

Further experiments were performed to investigate whether latently infected neurones in the ganglia innervating the ear differ from those in distal ganglia in their ability to reactivate virus after explantation. We have been able to address this problem directly using the SC16 110lacZ virus to monitor induction of IE110 promoter activity, which can be used as a marker for virus reactivation. By staining individual ganglia for β-Gal activity at various times post-explantation, it was possible to demonstrate virus reactivation at all ganglionic levels (C2 to C6). However, IE110 promoter-driven gene expression occurred earlier, and in a greater number of neurones, within ganglia in which IE gene expression had been observed during acute infection than in those where IE gene expression had not been detected. By 48 h post-infection there was a tenfold difference in the number of reactivating neurones seen at the C3 and C4 levels as compared to the C5 and C6 levels. ISH analysis of numbers of LAT-expressing neurones in these two groups of ganglia showed an approximately twofold difference in the levels of latency established. Hence, in the explant system there appears to be a difference in the ability of latent viral genomes to reactivate from the primary and secondarily infected neurones.

Do these two populations of latently infected neurones differ in any way which might explain this phenomenon? Using the zosteriform model of infection, approximately 200 copies of viral DNA per LAT-expressing neurone are detected in latently infected ganglia innervating the site of infection, whilst in distal ganglia the genome copy number is only 20 (Slobedman *et al*., 1994). In agreement with the findings of Sawtell *et al*. (1998), the data reported here suggest that virus may reactivate more efficiently from neurones containing a high viral DNA copy number.

The existence of a sub-population of neurones which contain a high copy number of viral DNA is intriguing (Sawtell, 1997; Slobedman *et al*., 1994). In the mouse ear and zosteriform models of latency, the high copy number latently infected neurones are found in ganglia in which there was detectable IE gene expression during the acute phase of infection. There are experimental data which suggest that, in infected sensory neurones, some viral DNA amplification may be required before the cell becomes fully permissive for IE gene expression (Kosz Vnenchak *et al*., 1990, 1993; Nichol *et al*., 1996). It is possible that some sensory neurones may survive limited expression of IE and early genes, and that amplification of viral DNA could precede the establishment of latency. Alternatively, the high copy number seen in some latently infected neurones could reflect input genomes.

We believe that recombinant viruses such as the ones described here will be useful tools in studying virus pathogenesis *in vivo*. By linking reporter genes to specific viral promoters, it is possible to investigate how observations made using replication-defective mutant viruses relate to infection with fully replication-competent viruses. The use of sensitive reporter genes also allows the activity of specific promoters during latency establishment and reactivation to be monitored within single cells in whole-mounted tissue.

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