Expression of β-galactosidase in neurons of dorsal root ganglia which are latently infected with herpes simplex virus type 1

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Explanation into culture of dorsal root ganglia (DRG) latently infected with herpes simplex virus type 1 (HSV-1) causes reactivation of the virus. Previous studies have suggested that either latency-associated transcripts (LATs) were removed as an early consequence of reactivation or, alternatively, there was a population of latently infected cells which did not contain LATs. We have now attempted to detect this population of neurons by inserting a reporter gene (Escherichia coli lacZ gene), under the control of promoters other than LAT, into the HSV-1 strain 17 mutant in1814, which was used in the earlier studies. One of these promoters, the human cytomegalovirus enhancer, resulted in weak expression of β-galactosidase in DRG neurons for at least 5 months.

The pattern of staining was predominantly homogeneous in neurons at 3 or 5 days post-infection or at 3 days post-explantation, and was predominantly speckled in latently infected neurons (1 to 5 months post-infection). About 30% of the β-galactosidase-positive neurons did not contain LATs by in situ hybridization. However, the detergents used to enable penetration of the substrate for β-galactosidase had also reduced the levels of the LATs; in neurons which originally had only small numbers of LATs this may have reduced levels to below those detectable by the methods used. There was, therefore, no unequivocal evidence for a population of latently HSV-1-infected cells which did not express LATs.

During latent infection with herpes simplex virus type 1 (HSV-1), viral genes associated with lytic replication are apparently not expressed but, in cells such as peripheral sensory neurons, RNAs called latency-associated transcripts (LATs) are expressed from the long repeat region of the genome (Dobson et al., 1989; Mitchell et al., 1990; Zwaagstra et al., 1990). The more stable RNAs of approximately 2.0 and 1.5 kb accumulate in the nuclei of latently infected cells (for review, see Fraser et al., 1992) where their presence can be demonstrated using in situ hybridization (ISH). The actual role of LATs in latent infections remains controversial but they have often been used as markers for latently infected cells.

Recent evidence has suggested that reactivation, induced by explantation of latently HSV-1-infected dorsal root ganglia (DRG) into culture, rarely occurred in cells which still expressed LATs (Ecob-Prince & Hassan, 1994). The LAT signal was detectable in other neurons of the same section, suggesting that the loss of LATs in neurons from which virus reactivated was not simply due to breakdown or turnover following explantation. The number of foci of reactivation at 3 days post-explantation was generally less than 3.0% of the estimated number of LAT+ cells in the same DRG. Explantation therefore stimulated reactivation of latent virus in very few latently infected cells. Furthermore, LATs were detectable in less than 1% of the cells in which virus reactivation was demonstrated. The question then arose as to whether virus reactivated from cells which did not express LATs or whether the LATs were removed as a prelude or early consequence of reactivation.

In an attempt to investigate whether there is a population of latently infected cells which do not express LATs we have constructed a virus, based on the in1814 genotype (Ace et al., 1989) used in the earlier studies, which also contains a reporter gene (β-galactosidase) under the control of a promoter independent of the LAT promoter. We have now investigated whether expression of the reporter gene from such a virus (called in1853) might act as a marker for the putative population of latently infected cells which do not express LATs.

The HSV-1 strain 17 mutant in1814 (Ace et al., 1989), containing a 12 bp insertion in the coding sequences for Vmw65, has been described previously by us in studies of herpesvirus latency (Ecob-Prince et al., 1993a, b). To analyse expression from different promoters, plasmids

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were constructed containing the *Escherichia coli* lacZ gene controlled by various promoters, inserted into the thymidine kinase (TK) or UL43 gene coding sequences. The plasmids were co-transfected with in1814 DNA and \( \beta \)-galactosidase-expressing viruses were identified by the development of blue plaques in the presence of X-gal; these were subjected to three rounds of plaque purification. The standard and purity of final virus preparations were confirmed by Southern hybridization, using radiolabelled probes which detected both the insert and parental sequences. In mutant in1853 a 760 bp Sau3AI fragment containing the human cytomegalovirus (HCMV) Towne strain enhancer (Stinski & Roehr, 1985) was cloned upstream of lacZ. The entire cassette was inserted into the TK gene at the unique SacI site. Virus titres were determined on BHK-21 cells in the presence of 3 mm-hexamethylene bisacetamide (McFarlane et al., 1992).

Three- to four-week-old male BALB/c mice were inoculated subcutaneously (0.025 ml containing approximately 8 \( \times \) 10^7 p.f.u. of virus) via the right rear footpad. At each time point [3 or 5 days, 1, 2 or 3 months post-inoculation (p.i.)], for each virus, the DRG from lumbar levels 2 to 6 were removed from six mice and fixed individually in 4% paraformaldehyde for 1 h at 4 °C. The DRG from three of the mice were then used for counts of \( \beta \)-galactosidase-positive (\( \beta \)-gal\(^+\)) neurons before being embedded in paraffin for serial sectioning; DRG from the other three animals were embedded directly. At 3 months p.i., DRG from an additional three mice were excised and explanted into culture for 3 days under conditions previously used to induce reactivation of latent TK\(^+\) viruses (Ecob-Prince et al., 1993b; Ecob-Prince & Hassan, 1994). These explanted DRG were then fixed and stained for the presence of \( \beta \)-galactosidase before being embedded in paraffin. To demonstrate the presence of \( \beta \)-galactosidase, fixed DRG were washed twice with PBS and incubated at 37 °C in a mixture containing 5 mm-potassium ferricyanide, 5 mm-potassium ferrocyanide, 2 mm-MgCl\(_2\), 0.02% NP40, 0.02% sodium deoxycholate and 1 mg/ml X-gal in PBS (X-gal medium). Each reaction was performed on a glass slide in a volume of 150 \( \mu \)l, with a 22 \( \times \) 40 mm coverslip sealed over the DRG with wax. At 3, 24 or 48 h the numbers of blue-stained cells were counted and the DRG photographed using a Zeiss Axioskop microscope with MC80 camera attachment. Serial sections of the embedded DRG from all the mice involved were then investigated by ISH for the presence of LATs or \( \beta \)-galactosidase mRNA. The ISH technique was carried out using \( ^{35} \)S-labelled riboprobes as described previously (Ecob-Prince et al., 1993a).

Expression of \( \beta \)-galactosidase was detected in neurons at 3 and 5 days p.i. with the viruses in1852, in1853, in1854 and in1855. Virus in1853 carries the HCMV enhancer; in1852 has the Moloney murine leukaemia virus (MoMLV) promoter plus enhancer, which is essentially a 760 bp EcoRI-Smal fragment from the long terminal repeat (LTR; Lang et al., 1983); in1854 contains the HSV-1 immediate early gene 1 promoter, an 845 bp BbvI–SacI fragment (Perry & McGeoch, 1988); in1855 contains the HSV-1 Vmw65 gene promoter, a 380 bp TaqI–EcoRV fragment from pMC1 (Dalrymple et al., 1985). Invariably, only DRG from lumbar regions 3, 4 and 5 contained positive cells, with the highest number usually in region 4, so the results presented here use the values from these ganglia, as suggested by Schmalfbruch (1987). At 3 days p.i. there were 110 \( \pm \) 11.0 \( \beta \)-gal\(^+\) neurons per mouse injected with in1853, 57 \( \pm \) 10.5 for in1852, 22 \( \pm \) 5.5 for in1854 and 77 \( \pm \) 5.0 for in1855. At 1 and 3 months p.i., although \( \beta \)-gal\(^+\) neurons were found in DRG latently infected with in1853 (23 \( \pm \) 4.6 and 43 \( \pm \) 16.3, respectively), none were found in DRG of mice infected with in1852, in1854 or in1855. Nevertheless, the number of LAT\(^+\) neurons, assessed at 3 months p.i. by ISH (143 for in1853; 161 for in1852; 155 for in1854 and 224 for in1855), indicated that all the viruses had established latent infections with similar efficiencies. Moreover, when DRG from these mice were explanted into culture for 3 days, \( \beta \)-gal\(^+\) neurons were once more detected (75 \( \pm \) 19.8 per mouse for in1853, 12 \( \pm \) 3.5 for in1852, 16 \( \pm \) 2.1 for in1854 and 17 \( \pm \) 4.2 for in1855) showing that virus was still present and able to express detectable levels of \( \beta \)-galactosidase under the right conditions.

The expression of \( \beta \)-galactosidase in animals injected with in1853, which gave detectable levels during latency, was therefore investigated in more detail. The pattern of \( \beta \)-galactosidase distribution in individual neurons varied, as observed by others (Ho & Mocarski, 1989; Dobson et al., 1990). We recognized a ‘homogeneous heavy’ stain which often extended to the axon of the neuron, a ‘homogeneous pale’ stain confined to the cell body, a ‘speckled’ distribution in which 5 to 20 foci of stain were observed in each cell, and a ‘speckled pale’ distribution in which the foci were mixed with patches of stain (Fig. 1a, b). To investigate the relationship between these patterns, DRG were photographed after 3 h or 21 h in X-gal medium and changes in the staining pattern of individual neurons were observed. The two basic patterns appeared to represent distinct distributions of enzyme rather than be the result of a simple quantitative difference. Moreover, the number of \( \beta \)-gal\(^+\) neurons after 3 h was, on average, 56% of that detected after 21 h. No further increase was observed if the reaction time was extended to 48 h, so counts were thereafter performed following incubation of ganglia for 24 h.

At various times after inoculation with in1853, or after
Fig. 1. Photomicrographs of β-gal + neurons in whole mounts of DRG (a, b) or in sections of DRG latently infected with in1853 (c, d, e). (a) Neurons with heavy (h) or speckled (s) staining patterns at 3 days p.i. (b) At 3 months p.i., neurons had mainly speckled patterns and a few had speckled pale (sp) patterns. (c, d) Sections which contain β-gal + neurons which do (c) or do not (d) also contain LATs. (e) LAT + neurons in DRG which have not been treated with X-gal prior to embedding. Bars represent 50 μm.

Table 1. The percentage of β-gal + cells which have different patterns of staining at different times after infection of mice or explantation of the DRG

<table>
<thead>
<tr>
<th>Time after injection or explantation*</th>
<th>Total number per mouse†</th>
<th>Homogeneous heavy</th>
<th>Homogenous pale</th>
<th>Speckled</th>
<th>Speckled pale</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days p.i.</td>
<td>107.5 ± 9.4</td>
<td>39</td>
<td>31</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>5 days p.i.</td>
<td>104.5 ± 14.2</td>
<td>27</td>
<td>40</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>1 month p.i.</td>
<td>40.6 ± 10.0</td>
<td>4</td>
<td>6</td>
<td>73</td>
<td>17</td>
</tr>
<tr>
<td>2 months p.i.</td>
<td>65.6 ± 11.5</td>
<td>14</td>
<td>25</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>3 months p.i.</td>
<td>37.7 ± 22.7</td>
<td>6</td>
<td>5</td>
<td>78</td>
<td>11</td>
</tr>
<tr>
<td>5 months p.i.</td>
<td>65.0 ± 22.6</td>
<td>1</td>
<td>0</td>
<td>86</td>
<td>13</td>
</tr>
<tr>
<td>3 days post-explantation</td>
<td>77.6 ± 15.3</td>
<td>46</td>
<td>34</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

* Mice were infected with approximately 8 x 10⁷ p.f.u. of in1853.
† Average for 3 to 8 mice per group.

explantation of latently infected ganglia, individual DRG were scored for the number of β-gal + neurons (Table 1). At 3 or 5 days p.i., just over 100 neurons per mouse contained β-galactosidase. This number had reduced by 1 month p.i. but was relatively stable thereafter at 2, 3 and 5 months p.i., with no evidence for a decline in the numbers of β-gal + neurons. At 3 days p.i., 90% of the β-gal + neurons were homogeneously stained but during
latex latency (1 to 5 months p.i.) the majority were of the speckled distribution. When DRG latently infected for 3 months were explanted into culture for 3 days to stimulate reactivation, the majority of cells expressing β-galactosidase did so with a homogeneous staining pattern. The homogeneous (usually heavy) pattern was therefore found to predominate at 3 and 5 days p.i. and at 3 days post-explantation, whereas the speckled pattern predominated at all times during latency (1, 2, 3 and 5 months p.i.).

To investigate whether persistence of β-galactosidase after infection with in1853 was due to the use of the TK gene as an insertion site, mutant in1891 (in which the UL43 gene was chosen as the insertion site) was constructed and tested. Mutants unable to express UL43 are known to be able to establish, maintain and reactivate from latency following injection of mouse pinnae (MacLean et al., 1991). Infection with in1891 resulted in numbers of β-gal+ cells similar to those in animals infected with in1853: 15 ± 3.1 at 1 months p.i., 32 ± 1.4 at 3 months p.i. and 67 ± 9.9 at 3 days post-explantation. The presence of enzyme was thus a consequence of the use of the HCMV enhancer rather than the site of insertion or the TK− phenotype of in1853.

The DRG in which the numbers of blue-stained β-gal+ neurons had been counted were embedded, serially sectioned and probed for the presence of LATs by ISH. The numbers of LAT+ nuclei and/or β-gal+ cells are presented in Table 2. Photomicrographs of β-gal+ neurons which contained (Fig. 1 c) or did not contain (Fig. 1 d) LATs in their nuclei are also shown. Of the LAT+ neurons present, only about 20% contained the blue stain in their cytoplasm, suggesting that the β-galactosidase cassette was only weakly active and was probably undetectable in most of the LAT+ cells. We were unable to regularly demonstrate mRNA for β-galactosidase by ISH in the blue-stained latently infected neurons, although the probe clearly detected the mRNA in lytically infected DRG (3 days p.i.) or BHK-21 cells (fixed at 24 h p.i.). Nevertheless, if all latently infected cells express LATs, it would be expected that all the blue-stained β-gal+ neurons should also contain LATs in their nucleus. It can be seen from Table 2 that only about 30% of the β-gal+ neurons did not contain LATs. It therefore appeared that the two markers were identifying different but overlapping populations of latently infected cells.

Table 2. The numbers of neuronal nuclei which contain LAT or β-galactosidase alone or both signals together

<table>
<thead>
<tr>
<th>Months after injection*</th>
<th>Total LAT+</th>
<th>Total β-gal+</th>
<th>β-gal+ LAT+</th>
<th>β-gal+ LAT-</th>
<th>β-gal+ LAT-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>13</td>
<td>39</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>116</td>
<td>14</td>
<td>106</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>21</td>
<td>49</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

* Mice were infected with in1853 and DRG from three to 12 animals were used for each time point.

However, as illustrated in Fig. 1 (c), the intensity of the autoradiographic grains associated with the LAT signal in these sections was always light (15–25 grains/nucleus) and did not demonstrate the medium or heavy densities reported before (Ecob-Prince et al., 1993a; Ecob-Prince & Hassan, 1994). Moreover, the grains were sometimes not confined to the nucleus but appeared scattered in the cytoplasm. The ISH for the LATs was therefore repeated on sections of latently infected DRG of sibling animals from the same experiments which had not been reacted with X-gal medium prior to embedding. The LAT signal in these sections showed the usual distribution of light, medium and heavy intensities of grains (Fig. 1 e). In addition, the numbers of LAT+ cells were consistently greater (at least double) in these DRG than in those which had been pretreated with X-gal medium. It was therefore concluded that the level of target RNA had been reduced following treatment with X-gal medium. This reduction still occurred when RNase-free X-gal medium and solutions for processing were used. The reduction appeared to be caused by the action of the detergents (NP40 and desoxycholate). These were necessary for penetration of the X-gal substrate into the DRG prior to sectioning but concomitantly had released some of the RNA signal from the cells.

The studies presented here show that when the HCMV enhancer was linked to the E. coli lacZ gene and inserted in either the TK (in1853) or UL43 (in1891) gene loci, β-galactosidase was detected in some latently infected neurons for at least 5 months p.i. However, despite this long-term expression, the HCMV enhancer appeared to act as a relatively weak promoter because β-galactosidase was not detected in all the latently infected LAT+ cells and we were unable to detect routinely its mRNA in the DRG. A failure to detect β-galactosidase mRNA has been reported by others (Margolis et al., 1993) but in a more recent report using the LAT promoter in conjunction with the MoMLV LTR linked to β-galactosidase, high levels of expression of both protein and mRNA were detected at 42 days p.i. (Lokensgard et al., 1994). The LTR alone did not, as in the present study, result in expression of β-galactosidase in latently infected DRG. Nevertheless, we conclude that the β-galactosidase detected during latency in mice infected with in1853 was not due to persistence of the protein expressed at 3 or 5 days p.i., for the following three reasons: (i) the other promoters used all resulted in expression of β-galactosidase at 3 days p.i. at levels equivalent to those found in animals infected with in1853, but no expression was detected at 1 month p.i. or later. (ii) The numbers of β-gal+ neurons in mice...
latently infected with in1853 showed no consistent decrease over 5 months. There was an initial fall from just over 100 per mouse at 3 or 5 days p.i. to between 40 and 64 at 1 to 5 months p.i. Although mRNA from immediate early, early or late genes could not be detected by ISH in trigeminal ganglia at 24 h p.i. (Kosz-Vnencak et al., 1990), expression of β-galactosidase from an immediate early or early promoter has been demonstrated at 2 and 4 days p.i. with a TK- virus (Ho & Mocarski, 1988). The expression of even low levels of immediate early genes might result in the death of the neurons (Johnson et al., 1992) and probably accounts for the drop in numbers of neurons between 5 days p.i. and 1 month p.i. (iii) The β-galactosidase protein has a half-life in most cell types which can be measured in hours (Margolis et al., 1993) and, although turnover in these cell systems is undoubtedly different to that in a non-replicating neuron, it seems unlikely that the protein would persist for 5 months unless sequestration in discrete compartments caused considerable stabilization. The speckled appearance of the β-galactosidase stain was predominant but not exclusive during latent periods of infection. The significance of this observation remains unclear but it could reflect a different metabolic environment within a neuron which can support latent infection.

Even though the HCMV enhancer appeared to result in long-term expression of β-galactosidase, it was not expressed in all LAT+-latently infected neurons. This could be because the LAT and HCMV promoters require different (but not necessarily mutually exclusive) sets of transcription factors which may not be present in neurons at all times. Alternatively, the HCMV enhancer may be active in all LAT+ cells but either at different levels or at levels which vary with changes in the intracellular environment, some of which are beneath detection by the methods used. The different levels of LAT at any time point seen by ISH in latently infected cells (Ecob-Prince & Hassan, 1994) might also suggest similar differences in the level of transcription of the LAT gene.

About 30% of the β-gal+ neurons did not also contain LATs. However, a proportion of the LAT signal appeared to have been lost as a result of the detergent treatment necessary for penetration of the X-gal substrate. Thus, the β-gal+ neurons which did not contain LATs may have been those which would otherwise have been classed as light grain intensity in autoradiography, previously shown to be about 28% of the total LAT+ neurons (Ecob-Prince & Hassan, 1994). Those β-gal+ neurons which also contained LATs (now of a light grain intensity in autoradiography) may previously have been ones with medium or heavy intensities. Recent studies by Ramakrishnan et al. (1994) demonstrated that LATs are expressed by only a minority of neurons harbouring the HSV-1 genome, and therefore at least some of the β-gal+ cells may genuinely support transcription from the HCMV enhancer but not the LAT promoter.

We thank Professor P. G. E. Kennedy and Professor J. H. Subak-Sharpe, in whose Departments this work was carried out, for their continued interest and support. Hazel Ireland and Mary-Jane Nicholl provided excellent technical assistance. The work was partly supported by a grant from the MRC to M. S. E.-P.

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(Received 5 December 1994; Accepted 7 February 1995)