Expression of the herpes simplex virus type 1 latency-associated transcripts does not influence latency establishment of virus mutants deficient for neuronal replication

M. P. Nicoll and S. Efstathiou

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1OP, UK

Herpes simplex virus type 1 establishes latency within neurons of the trigeminal ganglion. During latency, viral gene expression is largely restricted to the latency-associated transcripts (LATs), which, whilst not essential for any aspect of latency, function to suppress lytic gene expression and enhance the survival of virus-infected neurons. The latent cell population comprises primary-order neurons infected directly from peripheral tissues and cells infected following further virus spread within the ganglion. In order to assess the role of LAT expression on latency establishment within first-order neurons, we infected ROSA26R reporter mice with Cre recombiniase-expressing recombinant viruses harbouring deletion of the thymidine kinase lytic gene and/or the core LAT promoter. We found that LAT expression did not impact on latency establishment in viruses unable to replicate in neurons, and under these conditions, it was not required for the survival of neurons between 3 and 31 days post-infection.

Following primary mucosal infection, herpes simplex virus type 1 (HSV-1) gains access to sensory neurons of the trigeminal ganglion (TG) and establishes a latent infection. During latency, transcription of the virus genome is highly restricted and is largely limited to the latency-associated transcript (LAT), a non-protein-coding 8.3 kb mRNA encoded in repeat sequences flanking the U L region of the HSV-1 genome (Rock et al., 1987; Stevens et al., 1987). This primary transcript is processed into 1.5 and 2 kb stable introns (termed major LATs) (Zabolotny et al., 1997) and an unstable 6.3 kb (minor LAT) exon, the latter of which is further processed into numerous microRNAs (Jurak et al., 2010; Umbach et al., 2008). Whilst studies using small-animal models have demonstrated that LATs are not essential for latency (Javier et al., 1988; Steiner et al., 1989), these transcripts have been shown to influence the efficiency by which HSV-1 establishes (Nicoll et al., 2012; Perng et al., 2000b; Thompson & Sawtell, 1997) and/or reactivates from (Hill et al., 1990; Leib et al., 1989; Perng et al., 1994) latent infections. Furthermore, recent analyses have also provided evidence that the latent cell reservoir is less stably maintained (Nicoll et al., 2012) and loses long-term reactivation competence (Thompson & Sawtell, 2011) in the absence of LAT expression. The cellular and molecular basis of such LAT functions is less forthcoming, but is believed to centre on potential roles for the LAT in promoting cell survival (Branco & Fraser, 2005; Perng et al., 2000a; Thompson & Sawtell, 2001) and inhibiting immediate-early (IE) viral lytic gene expression by post-transcriptional and/or epigenetic mechanisms (Cliffe et al., 2009; Umbach et al., 2008; Wang et al., 2005). Indeed, in the absence of LAT expression, a greater frequency of TG cells expressing HSV-1 IE and early (E) gene transcripts can be observed by in situ hybridization during acute infection in mice (Garber et al., 1997), and increased lytic gene expression can also be detected during latency (Chen et al., 1997). It is not clear whether such an increase in virus lytic gene expression results in greater spread of the virus throughout neuronal tissue during acute infection, or indeed whether such a spread may result in an increased frequency of latently infected neurons. It has been found previously that HSV-1 LAT-negative mutants establish latency in fewer neurons relative to LAT-positive virus in the mouse TG (Thompson & Sawtell, 1997, 2001), a phenotype we have also observed following infection of the mouse whisker pad at high virus doses (Nicoll et al., 2012). In contrast to these data, we have also reported that HSV-1 recombinants deficient for LAT expression (due to deletion of the core latency-associated promoter, or LAP) established latency in ~33% more neurons per TG relative to revertant virus (following infection of mouse whisker pads with 10⁶ p.f.u.; Nicoll et al., 2012), suggesting that LAT expression may repress the replication and spread of HSV-1 within the TG. Despite this, we have been unable to observe LAT-dependent differences in virus replication within the TGs by standard assays of virus infectivity within BALB/c or ROSA26R mouse strains (Nicoll et al., 2012). The efficiency of latency...
establishment is influenced by numerous factors including: (i) the extent of peripheral replication; (ii) the number of primary-order neurons initially infected; (iii) the fate of infected neurons; and (iv) the extent of spread to neuronal cells within the ganglion that do not directly innervate the site of primary infection. Given the multitude of factors that can influence latent loads, we re-examined the establishment phenotype of LAT-positive and LAT-negative viruses with recombinants incapable of lytic replication within sensory neurons.

To examine the efficiency of latency establishment of LAT-positive and LAT-negative mutants in first-order neurons, we introduced a previously characterized deletion of the HSV-1 thymidine kinase (TK) gene (Fig. 1a) from HSV-1 SC16 recombinant TKDM21 (Efstathiou et al., 1989) into Cre reporter viruses HSV CMVCre [CMVCre carries the WT cre recombinase gene under the control of the major IE promoter and enhancer of human cytomegalovirus (CMV)], HSV CMVCreLAT–GFP (in which the 203 bp core LAP is replaced with a GFP expression cassette in the opposite orientation to the LAT transcript) and HSV CMVCreREV (a revertant virus of HSV CMVCreLAT–GFP) (Nicoll et al., 2012). HSV-1 mutants deleted for TK activity have been shown previously to replicate within epithelial tissues but are severely diminished or incapable of replication within sensory ganglia (Coen et al., 1989; Efstathiou et al., 1989; Thompson & Sawtell, 2000). Detection of lytic gene antigens during acute infection with TK-negative viruses suggests that this results from a deficit for viral DNA replication, as IE proteins, but not true late gene proteins, can be detected within mouse TGs (Sawtell et al., 2006). Thus, by inhibiting viral DNA replication in the sensory neurons of the TG, but not in cells at the periphery, the role of virus replication with regard to the frequency of latency establishment was assessed for both LAT-positive and LAT-negative viruses.

![Fig. 1.](image-url)
To generate the recombinant viruses HSV CMVCre TK⁻, HSV CMVCreΔLAT–GFP TK⁻ and HSVCMVCreREV TK⁻, infected-cell DNA prepared from HSV CMVCre, HSV CMVCreΔLAT–GFP and HSV CMVCreREV, respectively, was co-transfected into baby hamster kidney (BHK) cells with Scal-linearized pUC19-TKMD21. To construct pUC19-TKMD21, PCR primers (forward, 5'--AAAAATAGGTTAGGTCTTCGG-3'; reverse, 5'--AAAAATAAGCTTGGTACGCTAGGTTAGG-3') directing amplification between WT HSV-1 nt 46055 and 48113 (GenBank accession no. NC_001806; McGeoch et al., 1988) were utilized to clone the mutant TK-coding sequence from HSV-1 TK-negative recombinant TKMD21 (Efstathiou et al., 1989) into pUC19. Recombinant progeny containing the TKMD21 sequence were enriched twice by infection at a low m.o.i. of 0.01 of BHK monolayers in Dulbecco's modified Eagle's medium containing 10% FCS and supplemented with 5 μg acyclovir ml⁻¹. Recombinants were then purified by three rounds of limiting dilution infection in the presence of acyclovir and selected by PCR for the presence of an 831 bp deletion in the TK-coding sequence (data not shown). The presence of a TK deletion in all three recombinants was confirmed by Southern blot hybridization (Fig. 1b). All recombinants were observed by PCR to have retained the corresponding LAP genotype of their parental viruses (Nicoll et al., 2012) (data not shown). The in vitro replication kinetics of HSV CMVCre TK⁻, HSV CMVCreΔLAT–GFP TK⁻ and HSV CMVCreREV TK⁻ were similar, as well as being indistinguishable from each parental virus (Fig. 1c) following infection of BHK cell monolayers at a low m.o.i. of 0.01.

The TKMD21 mutation was demonstrated previously to render HSV-1 incapable of replication in cervical dorsal root ganglia, whilst retaining the ability to replicate within inoculated ear tissue, albeit at reduced titres throughout the duration of acute infection (Efstathiou et al., 1989). To ascertain whether an analogous phenotype would be observed following whisker pad infection, BALB/c mice were inoculated under isoflurane anaesthesia with 10⁶ p.f.u. of TK-positive HSV CMVCre, HSV CMVCreΔLAT–GFP TK⁻, HSV CMVCreΔLAT–GFP TK⁻, HSV CMVCreREV or HSV CMVCreREV TK⁻ by light scarification with 10⁶ p.f.u. per whisker pad, as described previously (Nicoll et al., 2012). Whisker pads and TGs were dissected at 4 days post-infection (p.i.), homogenized and assayed for infectious virus on BHK cell monolayers. All TK-deletion viruses achieved similar titres within whisker pad tissues, but were ~1 log lower than TK-positive viruses (Fig. 2a). Together, these data demonstrate that in the absence of TK activity each recombinant was competent for replication in whisker pad tissues, but displayed attenuation that was consistent across all TK-negative viruses. In contrast, no infectious virus was recovered from the TGs of mice infected with HSV CMVCre TK⁻, HSV CMVCreΔLAT–GFP TK⁻ and HSV CMVCreREV TK⁻, whilst >1000 p.f.u. could be recovered from all three parental TK-positive viruses (Fig. 2a). To confirm these observations, the experiment was repeated in ROSA26R mice with HSV CMVCre, HSV CMVCreΔLAT–GFP TK⁻ and HSV CMVCreREV TK⁻ (five mice per virus). As with the BALB/c mice, TK-negative virus infectivity could not be detected within the TGs, whilst a mean (±SEM) of 4.2 × 10² ± 7.5 × 10² p.f.u. of TK-positive HSV CMVCre was recovered at 4 days p.i. (Fig. 2b). Again, all TK-negative virus titres were comparable within whisker pad tissues (Fig. 2b), confirming that lytic replication was restricted to whisker pad tissue, in agreement with a previous study in which infectious virus was only infrequently detected in the TGs (and at <8 p.f.u. per positive ganglion at 4 days p.i.) following snout inoculation with a TK-null mutant (Thompson & Sawtell, 2000).

Having confirmed that all three TK-deleted recombinants did not replicate to detectable levels within TGs, we next assessed infected-cell marking during acute and latent infection using the previously characterized ROSA26R reporter mouse model of HSV-1 infection (Proença et al., 2008; Wakim et al., 2008). In this model, the human CMV major IE promoter-controlled expression of Cre recombinase from the infecting virus leads to the permanent genetic marking of latently infected neurons within the reporter mouse. This is due to Cre-mediated excision of a loxP-flanked neomycin resistance gene cassette situated between the constitutive ROSA26 promoter and a downstream lacZ reporter gene within the transgenic mouse (Fig. 2c). As a result, lacZ is expressed constitutively from the mouse genome, allowing visualization and enumeration of latently infected cells following incubation with the substrate X-Gal (Fig. 2d). Analyses of individually marked neurons by PCR for viral DNA have shown that all marked cells are virus DNA positive, supporting the view that the frequency of marked neurons accurately reflects latent loading of sensory ganglia (unpublished observations). Thirty-six ROSA26R mice were inoculated under isoflurane anaesthesia with 10⁸ p.f.u. per whisker pad of HSV CMVCre TK⁻, HSV CMVCreΔLAT–GFP TK⁻ or HSV CMVCreREV TK⁻ in groups of twelve animals. At 3 days p.i., four mice from each group were killed and the TG tissues dissected. Both TGs from each mouse were pooled, fixed in ice-cold 4% paraformaldehyde for 90 min, rinsed with PBS and incubated overnight at 37°C in X-Gal, as described previously (Lachmann & Efstathiou, 1997; Proença et al., 2011). The TGs were then photomicrographed and marked cells were counted in a blinded manner to avoid experimental bias. At 3 days p.i., the mean number of marked cells was comparable for each virus, with 131.5 (range 105–214), 157.9 (range 94–260) and 143.8 (range 86–228) marked cells per TG for HSV CMVCre TK⁻, HSV CMVCreΔLAT–GFP TK⁻ and HSV CMVCreREV TK⁻, respectively (Fig. 2e). No significant divergence was observed among the three marked cell populations (P=0.83; Kruskal–Wallis test). The remaining eight mice per group were killed at 31 days p.i. (a time point consistent with latency) and the TGs were dissected and treated as above. Once again, the marked cell...
populations were comparable among all three viruses; 137.2 (range 88–292), 166.9 (range 89–262) and 146.6 (range 99–206) marked cells per TG for HSV CMVCre TK–, HSV CMVCreΔLAT–GFP TK– and HSV CMVCreREV TK–, respectively (Fig. 2e), and no significant difference was observed between the three marked cell populations (P=0.41; Kruskal–Wallis test). Marked cell populations were highly comparable in three further independent experiments (Table 1). The mean of each marked cell population was reduced by fourfold to fivefold compared with analogous experiments with each TK-positive parental virus (Nicoll et al., 2012). Such differences are similar to those reported by Thompson & Sawtell (2000), in which the percentage of TG neurons latently infected by an HSV-1 TK mutant were approximately sixfold less than rescued virus following snout inoculation. Furthermore, in comparison with our previous data in which TK-positive LAT-negative viruses established latency in ~33% more neurons relative to LAT-revertant virus (at an infectious dose of 10^6 p.f.u. per whisker pad; Nicoll et al., 2012), the absence of an establishment phenotype in
Fig. 2. (a) Titres of TK-positive (HSV CMVCre, HSV CMVCreΔLAT–GFP and HSV CMVCreREV) and TK-negative (HSV CMVCre TK−, HSV CMVCreΔLAT–GFP TK− and HSV CMVCreREV TK−) recombinants within BALB/c whisker pad and TG pairs at 4 days p.i. (b) Titres of TK-positive (HSV CMVCre) and TK-negative (HSV CMVCre TK−, HSV CMVCreΔLAT–GFP TK− and HSV CMVCreREV TK−) recombinants within ROSA26R whisker pad and TG pairs at 4 days p.i. In (a) and (b), each symbol represents the titre from an individual mouse and the floating bar displays the mean titre from each virus-infected group. (c) Structure of the ROSA26 locus in R26R reporter mice. The transgene contains a splice acceptor sequence upstream of a neomycin phosphotransferase gene (neo) and four poly(A) (PA) signal sequences (together flanked by loxP sites) and a downstream lacZ gene. Following Cre-driven recombination of the loxP-flanked cassette, lacZ is constitutively expressed by the ROSA26 promoter. (d) Photomicrographs demonstrating the presence of marked cells following dissection, fixation and incubation of TGs with X-Gal. (e) Infected-cell marking from ROSA26R mice at 3 and 31 days following infection with HSV CMVCre TK−, HSV CMVCreΔLAT–GFP TK− and HSV CMVCreREV TK−. Each symbol represents the number of marked cells in an individual TG and the floating bar displays the mean number from each virus-infected group.

Table 1. Marked cell populations during latent infection with TK-negative HSV-1 recombinants in four independent experiments

<table>
<thead>
<tr>
<th>Experiment no. (days p.i.)*</th>
<th>Mean marked cell number per TG (range)</th>
<th>P†</th>
<th>n‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMVCre TK−</td>
<td>CMVCreΔLAT–GFP TK−</td>
<td>CMVCreREV TK−</td>
</tr>
<tr>
<td>1 (33)</td>
<td>130.9 (44–181)</td>
<td>127.1 (100–226)</td>
<td>141.4 (82–226)</td>
</tr>
<tr>
<td>2 (31)</td>
<td>137.2 (82–292)</td>
<td>166.9 (70–273)</td>
<td>146.6 (69–206)</td>
</tr>
<tr>
<td>3 (29)</td>
<td>–</td>
<td>113.1 (53–202)</td>
<td>77.7 (37–202)</td>
</tr>
<tr>
<td>4 (28)</td>
<td>89.6 (37–171)</td>
<td>89.1 (38–151)</td>
<td>–</td>
</tr>
<tr>
<td>All (28–33)</td>
<td>115.6 (37–292)</td>
<td>123.2 (38–273)</td>
<td>121.2 (37–226)</td>
</tr>
</tbody>
</table>

*Days p.i. at which marked cell numbers were assessed.
†P value ascertained from non-parametric analyses: Kruskal–Wallis for group-wise comparisons and Mann–Whitney for pairwise comparisons.
‡Number of individual TGs assessed from each virus group.

Acknowledgements

This research was supported by the Medical Research Council (MRC) UK (project grant PKAG/588). M. P. N. is supported by an MRC (UK) Centenary Award (project grant PKAG/588). All animal experiments were approved by the University of Cambridge ethical review board and by the UK Home Office under the 1986 Animal (Scientific Procedures) Act as Project Licence number 80/2205.
References


