The UL4 gene of herpes simplex virus type 1 is dispensable for latency, reactivation and pathogenesis in mice

Patricia Y. Jun,1 Lisa I. Strelow,1† Ronald C. Herman,2 Howard S. Marsden,3 Truls Eide,4 Lars Haarr4 and David A. Leib1,5

1,5 Department of Ophthalmology and Visual Sciences1 and Department of Molecular Microbiology5, Washington University School of Medicine, 660 South Euclid Avenue, Box 8096, St Louis, MO 63110, USA
2 Calydon Inc., Menlo Park, CA 94025, USA
3 MRC Virology Unit, Institute of Biomedical and Life Sciences, University of Glasgow, Church Street, Glasgow G11 5JR, UK
4 Centre for Research in Virology, University of Bergen, Bergen High Technology Centre, N-5020 Bergen, Norway

The UL4 gene of herpes simplex virus type 1 is predicted to encode a 21±5 kDa protein of 199 amino acids. Although UL4 is dispensable for growth in cell culture, its function is not known. In the present study, the promoter of UL4 was examined and found to contain a cAMP-response element which bound the transcription factor CREB, and was strongly activated by cAMP. A recombinant virus, termed UL4HS, was constructed with a nonsense linker inserted into the UL4 open reading frame, to make a truncated UL4 protein of 60 amino acids. In addition, a marker-rescued virus, UL4HSMR, was constructed. Western immunoblot analysis revealed a 23 kDa band in extracts of wild-type and marker-rescued virus infected cells which was missing for UL4HS. Only modest differences were observed in the abilities of wild-type and UL4-mutant viruses to grow in Vero cells or in contact-inhibited mouse C3H/10T1/2 cells. In addition, there were only modest differences between the ability of UL4HS to replicate in murine corneas and trigeminal ganglia relative to wild-type viruses, and reactivation of UL4HS from latency was unaffected. Taken together, these data demonstrate that UL4 is dispensable for latency and pathogenesis in mice.

Introduction

In humans and in experimental animals the pathogenesis of herpes simplex virus type 1 (HSV-1) occurs in a series of discrete stages (Wildy et al., 1982). Acute virus replication at the periphery is followed by virus entry into neuronal termini. Intra-axonal transport moves the virus to sensory ganglia, where further replication may occur and latency is established. The viral genome is episomal within neuronal nuclei, and the only abundant viral gene products expressed during latency are the latency-associated transcripts (Stevens et al., 1987). Latency may periodically break down in response to certain stimuli, leading to virus reactivation and shedding at the periphery. The molecular mechanisms responsible for the altered regulation of viral gene expression during latency remain poorly understood. A number of studies, however, have indicated that increased intracellular cAMP levels during latency may play a direct role in triggering the expression of a variety of cAMP-responsive viral genes, thereby leading to reactivation (Bloom et al., 1997; Deb et al., 1993; Leib et al., 1991; Wheatley et al., 1992). A perfect consensus cAMP-responsive element (CRE) exists 124 bp upstream of the UL4 gene, 64 bp upstream from its putative TATA box, although it is not known if this CRE is functional.

HSV-1 encodes at least 74 proteins, but the function of many of them remains unknown (McGeoch & Schaffer, 1993). Targeted mutagenesis of HSV-1 has demonstrated that many such genes are dispensable for virus replication in cell culture (Baines & Roizman, 1991). Experiments in which viruses with mutations in dispensable genes are used in animal models of neurovirulence, latency and reactivation have revealed that
many such genes play pivotal roles in the virus life-cycle in vivo. The use of stage-specific assays for the assessment of the pathogenesis of virus mutants in mouse models has allowed the determination of points in the life-cycle of the virus at which these genes play a role. For example, UL41 has been shown to be important for the establishment of latency (Strelow & Leib, 1995), ICP34.5 for replication in the nervous system (Taha et al., 1989a, b; Chou et al., 1990), and ICP0 and thymidine kinase for the reactivation of latency (Clements & Stow, 1989; Leib et al., 1989a; Coen et al., 1989); the LATs have been shown by various groups to be important for establishment and reactivation of latency (Leib et al., 1989b; Sawtell & Thompson, 1992; Steiner et al., 1989).

The protein products of several HSV-1 genes dispensable for growth in tissue culture have not yet been identified nor have functions been ascribed to those genes (McGeoch & Schaffer, 1993). The function of the UL4 gene, the subject of this study, is not known. The UL4 open reading frames (ORFs) of HSV-1 and HSV-2 show approximately 76% identity of corresponding amino acids after alignment, and the ORF of HSV-1 and HSV-2 show approximately 76% identity of the UL4 recombinant or its marker-rescued virus to replicate in various tissue types in cell culture, and surprisingly only modest differences in the virulence of the deletion mutant in pathogenesis of virus mutants in mouse models has allowed the determination of points in the life-cycle of the virus at which these genes play a role. For example, UL41 has been shown to be important for the establishment of latency (Strelow & Leib, 1995), ICP34.5 for replication in the nervous system (Taha et al., 1989a, b; Chou et al., 1990), and ICP0 and thymidine kinase for the reactivation of latency (Clements & Stow, 1989; Leib et al., 1989a; Coen et al., 1989); the LATs have been shown by various groups to be important for establishment and reactivation of latency (Leib et al., 1989b; Sawtell & Thompson, 1992; Steiner et al., 1989).

The protein products of several HSV-1 genes dispensable for growth in tissue culture have not yet been identified nor have functions been ascribed to those genes (McGeoch & Schaffer, 1993). The function of the UL4 gene, the subject of this study, is not known. The UL4 open reading frames (ORFs) of HSV-1 and HSV-2 show approximately 76% identity of corresponding amino acids after alignment, and the ORF of HSV-1 UL4 shows 25% identity with varicella-zoster virus (VZV) gene 56, and 17% identity with pseudorabies virus (PRV) UL4 (Altschul et al., 1995). The only published study of UL4 showed that it is dispensable for HSV-1 replication in tissue culture (Baines & Roizman, 1991). In that study, yield of virus from one round of infection of a UL4-deleted virus was 3-fold lower than wild-type virus in Vero cells and the same as wild-type virus in BHK TK− cells. In addition, the UL4 ORF specifies a protein of 25 kDa, and is regulated as a true-late (γ2) non-structural gene (T. Eide, L. Haarr and others, unpublished results).

In this study, the promoter sequences of UL4 were shown to be responsive to cAMP. In addition, a recombinant virus was generated in which a nonsense linker containing stop codons in all three reading frames was inserted into the UL4 gene such that only the amino-terminal third of the protein is expressed. There were no differences between the abilities of the UL4 recombinant or its marker-rescued virus to replicate in various tissue types in cell culture, and surprisingly only modest differences in the virulence of the deletion mutant in immunocompetent and immunodeficient mice. These studies demonstrate that UL4 is dispensable for replication in tissue culture and for virulence in mice.

**Methods**

**Cells and viruses.** African green monkey kidney (Vero) cells were propagated as described previously (Rader et al., 1993). Murine C3H/10T1/2 (ATCC CCL 226) were propagated in Dulbecco’s MEM supplemented with 10% foetal calf serum and antibiotics (250 U penicillin/ml and 250 µg streptomycin/ml) in a 3% CO2 humidified chamber at 37°C. Rat pheochromocytoma (PC12) cells (provided by Jeffrey Milbrandt, Washington University, USA) were propagated in a humidified incubator at 37°C with 12% CO2 in DMEM supplemented with 2 mM L-glutamine, 5% newborn calf serum and 5% horse serum (GIBCO-BRL). Growth and assay of the KOS strain of HSV-1 was done as described (Rader et al., 1993). Virus mutants UL4HS and UL4HSMR were propagated as described for KOS. One-step growth curve experiments were performed at 37°C on confluent Vero or C3H/10T1/2 cells as described (Strelow & Leib, 1995).

- **Transfections, CAT, gel retardation, and DNase I footprinting assays.** Transfection of PC12 cells and CAT assays were performed as described (Gorman et al., 1982). Where appropriate, 1 mM dibutyryl cAMP (dbcAMP), 0.5 mM isobutylmethylxanthine (IBMX) or 0.01 mM forskolin (Sigma) was added 18 h post-transfection and cells were incubated for a further 18 h before harvest and assay. DNA binding reactions and electrophoresis were performed for gel retardation assays as previously described (Leib et al., 1991). Eight ng of affinity purified CREB (provided by Marc Montminy, Salk Institute, USA) was added to 1 ng of 32P end-labelled p54-12 (see below) probe at 2–4 × 106 c.p.m./ng together with 50 ng of poly(dI-dC) as non-specific competitor. Wild-type specific competitor oligonucleotide was 5’ ACTAAATGACGTCGGCGC 3’ (CRE underlined) and mutant competitor was 5’ ACTAAATGACGGCTCGGCGC 3’ (altered nucleotides underlined). Nuclear extracts from PC12 cells were prepared and DNase I footprinting was performed as previously described (Lee et al., 1987, 1988). For probe preparation, p54-12 was cut with Xhol, and 200 ng of DNA was end-labelled with [32P]dCTP and [32P]dGTP using Klenow fragment. The resulting labelled DNA was cleaved with HindIII and the labelled fragment isolated on a 4% non-denaturing polyacrylamide gel and resuspended at 40 000 c.p.m./ml with 80 000 c.p.m. used per DNase I footprint.

- **Generation of recombinant plasmids and virus mutants.** The virus mutants used in this study were constructed from the parental strain KOS. Methods for constructing recombinants are described elsewhere (Rader et al., 1993). Enzymes were obtained from New England BioLabs and used according to the manufacturer’s instructions. A 3960 bp XhoI–BglII fragment was cut from the genome of strain KOS (positions 10636–14596; McGeoch et al., 1988) and ligated into the BamHI and XhoI sites of pGEM-7 (Promega) to yield plasmid pUL4. The entire UL4 open reading frame (positions 12424–11827) is within this fragment. Plasmid pUL4HS was constructed by the insertion of a nonsense linker (DeLuca & Schaffer, 1987) containing stop codons in all three reading frames as well as a unique HpaI site into the XcrI site at position 12226 in the open reading frame of UL4. pUL4HS was cotransfected with infectious KOS DNA into Vero cells, progeny virus was harvested and plaques were screened by Southern blotting as described for an altered HpaI digestion pattern using 32P-labelled pUL4 as a probe. Virus demonstrating the expected HpaI digestion pattern was plaque purified three times and a high titre stock was prepared and designated UL4HS. Marker-rescue to produce UL4HSMR was accomplished by cotransfection of infectious UL4HS DNA with a 208 bp EcoRI–SnaI fragment of KOS (positions 11084–13092) into Vero cells, followed by a low m.o.i. of C3H10T1/2 cells and screening by Southern blotting and plaque purifying as described for UL4HS. For CAT assays and DNase I footprinting procedures, the UL4 promoter region was cloned as a 147 bp EcoRI–SmaI fragment (positions 12593–12446) into pGEM-7 (Promega) to yield plasmid pUL4. The entire UL4 open reading frame (positions 12424–11827) is within this fragment. Plasmid pUL4HS was constructed by the insertion of a nonsense linker (DeLuca & Schaffer, 1987) containing stop codons in all three reading frames as well as a unique HpaI site into the XcrI site at position 12226 in the open reading frame of UL4. pUL4HS was cotransfected with infectious KOS DNA into Vero cells, progeny virus was harvested and plaques were screened by Southern blotting as described for an altered HpaI digestion pattern using 32P-labelled pUL4 as a probe. Virus demonstrating the expected HpaI digestion pattern was plaque purified three times and a high titre stock was prepared and designated UL4HS. Marker-rescue to produce UL4HSMR was accomplished by cotransfection of infectious UL4HS DNA with a 208 bp EcoRI–SnaI fragment of KOS (positions 11084–13092) into Vero cells, followed by a low m.o.i. of C3H10T1/2 cells and screening by Southern blotting and plaque purifying as described for UL4HS. For CAT assays and DNase I footprinting procedures, the UL4 promoter region was cloned as a 147 bp EcoRI–SnaI fragment (positions 12593–12446) into pGEM-7 (Promega) to yield plasmid p54-12. Introduction of these promoter fragments into plasmids for use in CAT assays was performed using standard procedures as described previously (Gorman et al., 1982).

- **Antiserum production and Western blotting.** An oligopeptide was synthesized that corresponded to the carboxy terminus of UL4 of strain KOS, namely QLSPFLLEYADKLLG5. The peptides were made as multiple antigenic peptides (Tam, 1988) as these have been shown to generate sera with higher anti-protein titres (McLean et al., 1991). Peptide
was used to raise antiserum in rabbits and Western blotting performed. Briefly, cell lysates were separated on a 12.5% acrylamide gel, transferred to Hybond ECL (Amersham) and preblocked in modified Denhardt’s solution before incubation with UL4 antiserum. Filters were washed with PBS–Tween and then incubated with AMDEX (goat anti-rabbit IgG, Amersham) before washing with PBS–Tween and overnight washing with PBS. Bands were visualized using an ECL kit (Amersham) and autoradiography.

Animal procedures. Outbred CD-1 female mice (21–25 g) (Charles River Breeding Laboratories, Kingston, NY, USA) were anaesthetized with ketamine and xylazine, their corneas bilaterally scarified and then incubated with UL4 antiserum. Filters were washed with PBS–Tween and then incubated with AMDEX (goat anti-rabbit IgG, Amersham) before washing with PBS–Tween and overnight washing with PBS. Bands were visualized using an ECL kit (Amersham) and autoradiography.

Results

CAMP and activity of the UL4 promoter region

A putative cAMP response element exists approximately 50 bp upstream of a consensus TATA box which, in turn, is 109 bp upstream of the initiating methionine of UL4 (Fig. 1). This CRE, which is an 8 base palindrome (5’ TGACGTCA 3’) is highly conserved with sequences from a wide variety of genes whose expression is regulated by cAMP (Montminy et al., 1990). CAT assays were therefore performed to examine the functionality of this element. The results shown in Fig. 2 show that basal activity of the UL4 promoter region is low in PC12 cells, but is induced 7-fold by the cAMP analogue 3’-O-dibutyryl cAMP, 6.5-fold by the phosphodiesterase inhibitor IBMX and 4.5-fold by the adenylate cyclase activator forskolin. All of these agents raise intracellular cAMP concentrations, although by different mechanisms. These results strongly suggest that the CRE within the UL4 promoter is indeed functional.

Transcriptional upregulation of promoters containing CREs occurs through the activity of cAMP-dependent protein kinase, which phosphorylates the transcription factor CREB and a family of related proteins. The binding to CREs of the cognate transcription factor CREB is therefore a further hallmark of cAMP-regulated promoters. As shown in Fig. 3, purified CREB binds specifically to the CRE in the 147 bp UL4 promoter region. CREB binding is competed in a dose-dependent fashion by an unlabelled 18-mer oligonucleotide which overlaps the CRE site (lanes 2–5), but not by up to a 100-fold excess of an oligonucleotide with a 3 bp alteration in the CRE site. To identify further areas in this promoter which are bound by proteins from nuclear extracts from PC12 cells, DNase I footprinting was performed. As shown in Fig. 4, strong protection was observed over the CRE itself and the region 4 nt immediately 3’ of the CRE. No other significant areas of protection were seen. Taken together, these data suggest that the CRE in the UL4 promoter region is responsive to cAMP, and that it specifically binds CREB and possibly other cellular factors.

Generation of recombinant virus mutants

In order to define the function of UL4, a recombinant virus containing a null mutation in UL4 was constructed. Potential recombinants were screened by picking of plaques and screening for an altered Hpa I digestion pattern (Figs 1 and 5a), with wild-type and marker-rescued virus giving an expected band of 6.4 kb, and the UL4 mutant (termed UL4HSMR) showing bands of 3.8 and 2.6 kb. Of 86 plaques screened on the first round of plaque selection, one plaque showed the appropriate pattern for a UL4 mutant. Three rounds of further plaque purification yielded a pure population of UL4HSMR which was grown to a high titred stock. To make the marker-rescued UL4HSMR virus, transfection progeny were passaged at a low m.o.i. through C H10T1/2 to help select for virus growing with wild-type kinetics since UL4HSMR grows approximately 5-fold less efficiently than wild-type on these cells. This strategy was successful since on the first round of random selection thereafter, one plaque of eleven showed the marker-rescued genotype, an enrichment of approximately 8-fold. Three rounds of further plaque purification yielded a pure population of UL4HSMR, which was grown to a high titred stock.

Western blotting was performed on cell extracts prepared from Vero cells that were mock-infected or infected with KOS, UL4HSMR or UL4HS (Fig. 5b). The rabbit antiserum used was raised to the carboxy-terminal 15 amino acids of KOS UL4 (predicted to be absent from UL4HSMR, which lacks the carboxy-terminal two-thirds of the protein). A protein of the approximate expected size for UL4 (21.5 kDa) was observed in both the KOS and UL4HSMR lanes, but not in lanes containing either UL4HS or mock-infected extracts.

Replication kinetics

Replication kinetics of wild-type and UL4HS viruses were examined in Vero, mouse C H10T1/2 and primary human foreskin fibroblast cells. KOS and UL4HS both replicated efficiently in Vero cells in a one-step growth curve and virus yields and growth kinetics were not significantly affected by mutation of UL4 at both high (10) and low (0·1) m.o.i. values.
Fig. 1. Map of the UL4 ORF showing the virus mutant generated in this study. (a) Prototypical arrangement of the HSV-1 genome, showing unique long (UL) and unique short (US) segments flanked by internal (a’, b’, c’) and terminal (a, b, c) repeats. UL4 is transcribed in a leftward direction, as indicated by the arrow. (b) Expanded view of UL4 region showing selected restriction enzyme sites. The numbering corresponds to that of McGeoch et al. (1988). (c) Wild-type UL4 ORF of 199 codons. (d) Mutant UL4 ORF created by the insertion of a nonsense linker containing a unique HpaI restriction site and stop codons in all three reading frames at the XcmI site.

Fig. 2. CAT assays of the UL4 promoter region, termed p54-12, in PC12 cells in the absence or presence of 1 mM dibutyryl (dB) cAMP, 0.5 mM isobutylmethylxanthine (IBMX) or 10 μM forskolin (Fs). The promoter was from positions 12593 to 12446 (McGeoch et al., 1988). Numbers above bars represent mean percentage conversion values from two experiments.

The two viruses showed similar replication in contact-inhibited mouse C5H/10T1/2 cells although there was a 3- to 5-fold decrease in UL4HS yields relative to KOS (Fig. 6b). Growth curves in human foreskin fibroblasts (data not shown) were identical to those seen for the C5H/10T1/2 cells. These results indicated that the growth of UL4HS was not severely restricted in human, non-human primate or mouse cells in culture.

The growth of KOS, UL4HS, and UL4HSMR was examined in mouse corneas and trigeminal ganglia 1–7 days post-infection following scarification and infection with 2 × 10^6 p.f.u. per eye. The replication of KOS, UL4HS and UL4HSMR were indistinguishable in eyes and ganglia over this time period (Table 1 and Fig. 7). Moreover, the extent of overt clinical signs (keratitis, blepharitis, hair loss) during acute infection, measured on a semi-quantitative scale, was not statistically different between KOS-, UL4HS- and UL4HSMR-infected mice, although over three experiments the UL4HS scores were consistently slightly higher than UL4HSMR or KOS (Table 1). In immunodeficient (SCID) mice, however,
there was no significant difference in the time to death at two different doses of UL4HS or UL4HSMR viruses following intraperitoneal infection (Table 1). Taken together, these data suggest that there are only slight differences in these viruses’ ability to replicate and to cause disease in vivo.

Reactivation studies

Initial studies of UL4HS reactivation were performed by explant cocultivation assays following an input dose of $2 \times 10^6$ p.f.u. per eye (Table 1). In these assays, virus from 21 of 26 (81%) of KOS latently-infected ganglia and 19 of 24 (79%) UL4HS-infected ganglia reactivated, suggesting no significant differences in their abilities to reactivate in this assay. Southern blotting of reactivated samples revealed that they were identical to input viruses, ruling out reversion or contaminated virus stocks (data not shown). Explant cocultivation assays, being an all-or-none assay, however, cannot assess the kinetics of reactivation. The kinetics of reactivation of KOS, UL4HS and UL4HSMR were therefore examined in dissociated ganglion cultures. By this method, there was no significant difference between any of the viruses with a mean time to 50% reactivation of approximately 3 days for an input of $2 \times 10^6$ p.f.u. per eye (Table 1). Maximal reactivation was accomplished by all viruses with comparable timing (data not shown). Reactivation therefore appears to be unaffected by the presence or absence of UL4.

Discussion

A previous study had demonstrated that UL4 is non-essential for replication in tissue culture (Baines & Roizman, 1991). Many genes which have been shown to be non-essential for replication in cell culture have subsequently been shown to play an important role in latency and pathogenesis. The presence of a perfect consensus CRE motif upstream of UL4, a motif which has been implicated in the regulation of several viral genes which may be important for pathogenesis, further suggested that UL4 may be important in this respect. (Deb et al., 1993; Leib et al., 1991; Wheatley et al., 1992.) The region upstream of UL4 is strongly responsive to cAMP, binds CREB specifically, and binds nuclear factors strongly in the region of the CRE. This study, however, clearly demonstrates that UL4 itself is not required for virulence in CD-1 mice or lethality in SCID mice. Another UL4 mutant, R7217, showed a 13-fold attenuation in intracranial injections of mice with an LD$_{50}$ of 610 p.f.u. compared to an LD$_{50}$ of 46 p.f.u. with wild-type virus (J. Baines & B. Roizman, personal communication). While a 13-fold drop in LD$_{50}$ may be significant in that unpublished study, it is clear that deletion of UL4 does not result in a drastic change in virulence as seen for other genes (Jacobson et al., 1989; Leib et al., 1989a; Taha et al., 1989a, b; Chou et al., 1990; Strelov & Leib, 1995). There are several possible explanations for these observations. First, that UL4 is a vestigial gene whose function is now lost. Second, that the mutation in UL4 was insufficient to destroy function. Third, that UL4 has a function in human pathogenesis which cannot be ascertained in a mouse model. Fourth, that UL4 has a function which was not addressed in any of the assays of this study. These possibilities will be discussed in turn.

First, the idea of UL4 being a vestigial gene should be considered as most unlikely since the gene is conserved in all alphaherpesviruses. The HSV-1 UL4 protein is 76% identical to UL4 of HSV-2, and 25% identical to gene 56 of VZV. Homologies to other herpesviruses include 17% identity to bovine herpesvirus and PRV proteins, and 15% and 12% identities to gallid and canine herpesvirus proteins (Altschul et al., 1990). The homology of 25% with gene 56 of VZV is significantly below average for these two genomes and homologies with all viruses, with the exception of HSV-2, are limited (Dean & Cheung, 1994). Sequence similarity scores,
however, are significantly higher by CGC BestFit analysis (Vlcek et al., 1995) and there are many examples of HSV-1 genes with strong similarity to HSV-2 genes but limited homology to other herpesviruses, where the genes are clearly functional. On the basis of sequence homologies, it is therefore unlikely that UL4 is an ancestral gene for which the function has been lost.

Second, there is the possibility that the mutant form of UL4 is still functional. This is also considered to be unlikely. The UL4HS UL4 protein is predicted to be truncated after 60 amino acids, the full-length protein being 199 residues. UL4HS therefore encodes only 33% of the wild-type protein. The only other published UL4 mutant, termed R7217 (Baines & Roizman, 1991), encodes 49% of the UL4 protein and was used to demonstrate that UL4 was dispensable for growth in cell culture, although no analysis of the protein encoded was performed. The only observable phenotypic difference in this study was the slight growth defect observed in culture, which is consistent with the data for R7217 (Baines & Roizman, 1991). The likelihood is that UL4 function has been destroyed in both recombinant viruses.

Third, it is possible that UL4 has a function in human pathogenesis which cannot be ascertained in a mouse model. This situation has precedent in herpesvirology with ICP47, which interferes with antigen presentation by interaction with human TAP, but does not interact with the murine homologue.
Table 1. Acute replication, clinical scores and reactivation of latency for KOS, UL4HS and UL4HSMR

<table>
<thead>
<tr>
<th>Virus</th>
<th>Eye swabs*</th>
<th>Ganglia*</th>
<th>Clinical score†</th>
<th>SCID mortality‡</th>
<th>Explant reactivation§</th>
<th>Reactivation kinetics¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>$8.7 \times 10^3$</td>
<td>$1.0 \times 10^4$</td>
<td>2.1</td>
<td>ND</td>
<td>21/26</td>
<td>2.8</td>
</tr>
<tr>
<td>UL4HS</td>
<td>$1.1 \times 10^3$</td>
<td>$4.2 \times 10^4$</td>
<td>2.6</td>
<td>8.1</td>
<td>19/24</td>
<td>2.5</td>
</tr>
<tr>
<td>UL4HSMR</td>
<td>$3.4 \times 10^3$</td>
<td>$4.3 \times 10^4$</td>
<td>2.1</td>
<td>7.5</td>
<td>ND</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

* Mean virus titres (p.f.u./ml) on day 3 following infection with $2 \times 10^6$ p.f.u. per eye.
† Mean clinical score per group according to a semi-quantitative scale of 1 to 4.
‡ Overall mean time in days to death for SCID mice following intraperitoneal infection with either $8 \times 10^7$ p.f.u. (five mice) or $3 \times 10^8$ p.f.u. (five mice).
§ Number of explanted latently infected trigeminal ganglia yielding virus per total number of ganglia assayed.
¶ Mean time in days to reactivation of 50% of neuronal cultures.
ND, Not determined.

Fig. 7. Acute virus replication in mice. Logarithmic mean titres of KOS, UL4HS and UL4HSMR in mice following corneal inoculation with $2 \times 10^6$ p.f.u. per eye of virus. (a) Eye swabs were taken at 3 h, 1, 2, 3, 5 and 7 days post-infection. (b) Trigeminal ganglia were removed and assayed directly for infectious virus at 3, 5 and 7 days post-infection.

(Tomazin et al., 1996). Clearly, it is not possible to extrapolate any information in this regard from the present study although it is likely that many aspects of HSV pathogenesis in humans cannot be modelled in rodents. One approach to this problem could be to generate recombinants of UL4 homologues in other viruses such as PRV which can then be tested in their natural host.

Fourth, the appropriate in vitro assay may not have been employed in this study. Although six different aspects of pathogenicity were measured in several independent experiments (replication in eyes, ganglia, clinical scores, explant reactivation, dissociated ganglion reactivation kinetics, SCID mouse lethality) no difference in phenotype was detected. Perhaps by use of an in vitro reactivation model a phenotype could be determined. In the example of certain LAT deletion mutants a more profound phenotype of reduced reactivation is seen (Hill et al., 1990; Perng et al., 1994; Sawtell & Thompson, 1992) than in explant cocultivation, in which the system is heavily biased towards reactivation. Such mutants, however, do always have a discernible altered phenotype in explant assays (Leib et al., 1989b; Rader et al., 1993; Steiner et al., 1989). Indeed, there are no published examples of viruses which have altered phenotypes by an in vitro reactivation system but no altered phenotype in an explant model. Although the UL4 promoters of both HSV-1 and HSV-2 contain perfectly matched palindromic CREs, elements which have been implicated in the reactivation process in several types of latency models (Rader et al., 1993; Bloom et al., 1997), these studies suggest that it is unlikely that UL4 plays any major role in pathogenesis, at least in murine models. This challenges the assumption that HSV genes which are non-essential for replication in cell culture must play a role in pathogenesis. Clearly, a role for UL4 in the HSV life-cycle remains to be determined.

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