Journal of NeuroVirology, 12: 5–16, 2006

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280500516401

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A mutant deleted for most of the herpes simplex virus type 1 (HSV-1) UOL gene does not affect the spontaneous reactivation phenotype in rabbits

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The mechanisms involved in the herpes simplex virus type 1 (HSV-1) latency-reactivation cycle are not fully understood. The latency-associated transcript (LAT) is the only HSV-1 RNA abundantly detected during neuronal latency. LAT plays a significant role in latency because LAT(−) mutants have a reduced reactivation phenotype. Several novel viral transcripts have been identified within the LAT locus, including UOL, which is located just upstream of LAT. The authors report here on a mutant, ΔUOL, which has a 437-nucleotide deletion that deletes most of UOL. ΔUOL replicated similarly to its wild-type parental McKrae HSV-1 strain in infected cells, the eyes, trigeminal ganglia, and brains of mice and rabbits. It was indistinguishable from wild-type virus as regards explant-induced reactivation in mice, and spontaneous reactivation in rabbits. In contrast, ΔUOL was significantly less virulent in mice. Thus, UOL appears to be dispensable for the wild-type reactivation phenotype while appearing to play a role in neurovirulence in ocularly infected animals. *Journal of NeuroVirology* (2006) **12,** 5–16.

Keywords: HSV-1; LAT; reactivation; UOL; virulence

Introduction

Herpes simplex virus type 1 (HSV-1) is ubiquitous HSV-1 infection and is characterized by two discreet phases: lytic and latent (Whitley and Roizman, 2001). Lytic infection, in which the host cell is killed and progeny virus is released, occurs at and near the site of viral entry. Following peripheral infection and replication, HSV-1 travels through the axons to the nuclei of sensory neurons of the trigeminal ganglion (TG) where the virus establishes life long latency in its host in the absence of clinical manifestations (Stevens and Cook, 1971). However, periodically, due to factors such as hormonal changes, stress, and ultraviolet (UV) exposure, the latent virus reactivates, causing secondary infection and clinical symptoms at or near the site of initial infection (Whitley and Roizman, 2001). Recurrent HSV-1 corneal infection can lead to scarring of the cornea and blindness (Roizman and Sears, 1996).

Lytic infections are mainly studied in tissue culture and have been shown to result in synthesis of more than 80 different viral gene products (Roizman and Sears, 1996). Latent infections have usually been studied in animal models where more limited gene

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This work was supported by Research to Prevent Blindness, The Discovery Fund for Eye Research, The Henry L. Guenther Foundation, Public Health Service grant NIH EY13191, and UCI COM grants. Dr. Wechsler is an RPB Senior Scientific Investigator. The authors thank Jennifer Cooper for her technical advice in mouse TG explant reactivation assays. They also thank Anita Avery for her excellent assistance with tissue culture and virus titrations.

Received 16 July 2005; revised 10 October 2005; accepted 25 November 2005.

expression occurs (Jones, 2003). The host and viral factors that influence the establishment, maintenance, and ultimately reactivation of HSV-1 have been difficult to determine.

During latency no infectious virus can be recovered, although a limited number of viral transcripts have been detected in latently infected neurons (Roizman and Sears, 1996). Of these, the latency-associated transcript (LAT) is the only viral gene product that accumulates to a high enough degree in neurons to be detected by Northern blot and *in situ* hybridization analysis (Deatly *et al*, 1988; Rock *et al*, 1987; Spivack and Fraser, 1987; Stevens *et al*, 1987; Wagner *et al*, 1988). LAT, encoded in the long internal repeats of the viral genome, can be thought of as a family of transcripts derived from the single primary LAT transcript. The primary LAT transcript is about 8.3 kb in length, overlaps infected cell protein zero (ICP0) in an antisense manner (Perng *et al*, 1996b; Stevens *et al*, 1987) and is very difficult to detect. A 2.0-kb LAT, a hallmark of neuronal latency, appears to be an intron spliced from the 8.3 kb primary transcript (Farrell *et al*, 1991). This 2-kb LAT accumulates in latently infected neurons and is readily detectable. The core TATAA box of the primary LAT promoter is located near nucleotide 118,773 of the viral genome (Perng *et al*, 1994c; Zwaagstra *et al*, 1989). Viruses lacking this promoter fail to accumulate LAT transcripts in infected cells (Perng *et al*, 1994a).

Although the mechanisms of LAT's function has remained elusive since its discovery (Rock *et al*, 1987; Stevens *et al*, 1987), it is clear that LAT is essential for the wild-type (high) reactivation phenotype because LAT mutants reactivate poorly (Bloom *et al*, 1996; Leib *et al*, 1989; Perng *et al*, 1994a; Steiner *et al*, 1994). We reported that LAT has antiapoptotic activity (Perng *et al*, 2000) and this activity maps to the first 20% of the primary LAT transcript, the same region that is sufficient for supporting the wild-type reactivation phenotype LAT3.3A (Perng *et al*, 1996b). No LAT proteins have been identified from this region, although a LAT protein encoded downstream of this region has been reported (Thomas *et al*, 1999, 2002). The mechanism mediating the reactivation phenotype is currently unknown, although it appears that LAT's antiapoptosis activity plays an important role (Ahmed *et al*, 2002; Ciacci-Zanella *et al*, 1999; Perng *et al*, 2000).

Several novel transcripts within the LAT promoter region have been reported (Singh and Wagner, 1993). Two HSV mutants with a portion of these transcripts deleted have wild-type reactivation phenotypes in the mouse explant cocultivation assay (Maggioncalda *et al*, 1996; Zhu *et al*, 1999). More recently, a protein, UOL (Upstream of LAT), encoded in the LAT promoter regulatory region, has been identified and characterized (Naito *et al*, 2005). However, these studies did not look into the effects of the deletion on spontaneous reactivation or neurovirulence.

In this report, an HSV mutant (ΔUOL) was constructed in HSV-1 strain McKrae and the effects of this UOL deletion on the spontaneous reactivation phenotype in infected rabbits was analyzed.

Results

Genomic structure of ΔUOL

We previously reported that the UOL gene is located upstream of the LAT promoter region (Naito *et al*, 2005). A schematic drawing of the relative location of UOL in the HSV-1 genome is shown in Figure 1. The UOL gene is located within the HpaI P fragment (Figure 1B). Its location relative to LAT is shown in Figure 1C. By removing 437 nuclotides (nt) corresponding to genomic nt 118,003 to 118,442 (Figure 1E), and the corresponding location in the terminal long repeat, a majority of the UOL open reading frame and the 5′ end of transcript was deleted (Figure 1D).

The 437-nucleotide deletion in the ΔUOL viral genome was confirmed by Southern analysis (Figure 2A). Purified viral McKrae, dLAT2903, or ΔUOL DNAs were digested with either BamHI or HpaI restriction enzyme, separated on an agarose gel, transferred onto a membrane, and hybridized with a UOL specific probe (HSV-1 genomic nt 118,266 to 118,711) labeled with ^{32}P isotope. When the viral DNA was digested with BamHI and probed with the UOL-specific gene, two DNA bands were observed (Figure 2A, BamHI). The BamHI digestion occurred not only at the repeat region but also cut at the U_L region of the viral genome, thus two bands were seen. The 437-nt deletion in ΔUOL resulted in little change in the BamHI digestion lane because large fragments of DNA were generated (~9 to 10 kb). In contrast, HpaI digestion produces smaller fragments (~2.8 to 3.2 kb), resulting in a readily detectable DNA fragment shift (Figure 2A, HpaI). The HpaI digestion covers the repeat and U_L region, thus two DNA bands are seen. In dLAT2903, a LAT-null mutant, a large region of viral DNA (Perng *et al*, 1994a) including an internal HpaI site within the repeat regions, was removed. Thus, with BamHI digestion, a DNA fragment shift was seen, whereas with HpaI digestion a single DNA band with high molecular weight was observed (Figure 2A).

Wild-type replication of ΔUOL in CV-1 cells

CV-1 cells were infected with ΔUOL, dLAT2903, or the parental wild-type (McKrae) virus at a multiplicity of infection (MOI) of 0.1 or 10 plaque forming units (PFU)/cell. Total virus was harvested at various times post infection and the amount of viral replication was determined by standard plaque assays. Replication of ΔUOL, appeared similar to that of McKrae and dLAT2903 at both MOIs (Figure 2B).

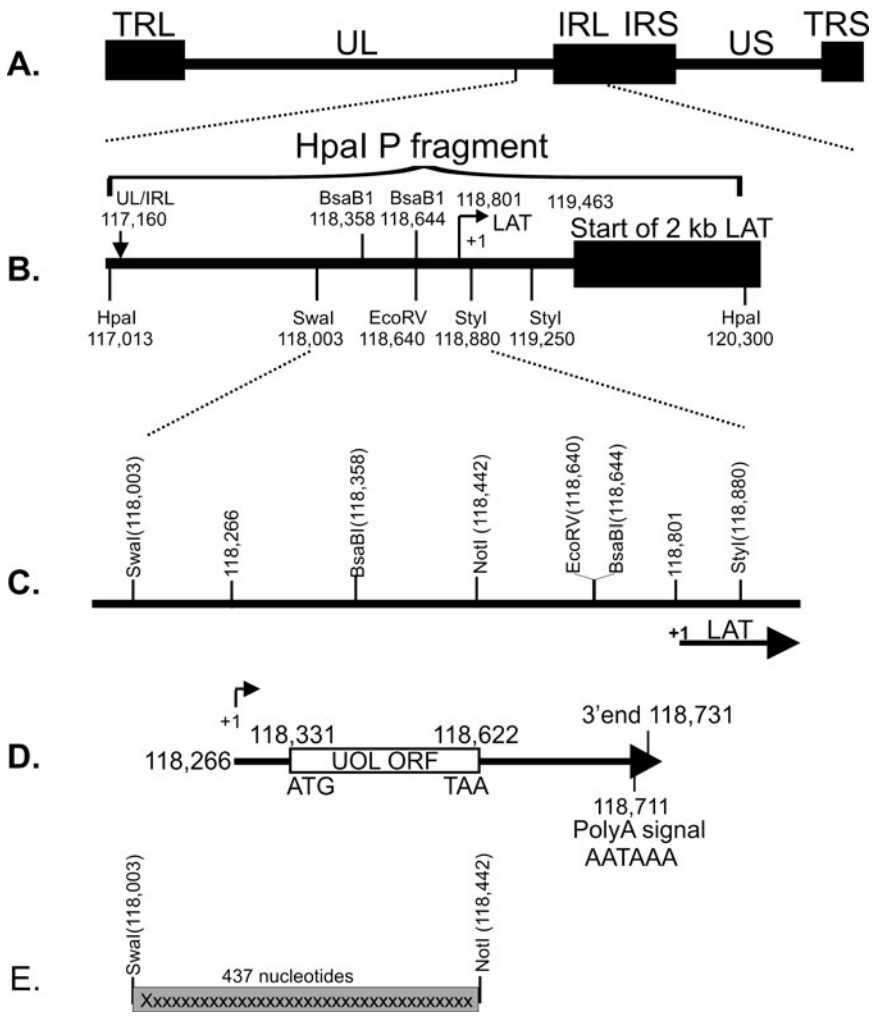

Figure 1 Schematic description of relative location of UOL. (**A**) Genomic structure of wild type HSV-1. TRL and IRL, terminal and internal long repeats, respectively; TRS and IRS, terminal and internal short repeats, respectively; UL and US, long and short unique regions, respectively. (**B**) Expanded region of HpaI P fragment. UL/IRL shows the location of the junction between the unique long and internal long repeats. The numbers refer to the HSV-1 genomic nucleotide positions for strain 17syn+ (McGeoch *et al*, 1988; Perry *et al*, 1998; Perry and McGeoch, 1988). The locations of several restriction enzyme sites are indicated. +1 is the start of the primary LAT transcript. The black rectangle box indicates the 5′ end of the stable 2-kb LAT. (**C**) Further expansion to show the detail of upstream of LAT. (**D**) The location of UOL mRNA shown in more detail. The UOL transcript starts at nucleotide 118,266 and the UOL open reading frame (ORF) begins at nucleotide 118,331 (ATG) and ends at nucleotide 118,622 (TAA). The polyA addition signal is at 118,711 and the 3′ end of the UOL mRNA is at nucleotide 118,731. (**E**) UOL deletion. The deleted area region is from nucleotide 118,003 to 118,442 (SwaI to NotI). The shaded rectangle filled with XXXX indicates the deletion in ΔUOL virus. Thus, the 437 nucleotides are deleted.

Expression of LAT in ΔUOL

To determine the effects of the ΔUOL 437-nt deletion on LAT expression, CV-1 cells were mock infected or infected with ΔUOL, dLAT2903, or McKrae at 5 PFU/cells. Total RNAs were isolated at 24 h post infection and Northern blotting was performed as described in Materials and Methods. The levels of LAT expression in ΔUOL were compatible to that of McKrae (the parental virus) at 24 h post infection (Figure 2C, *upper panel*). As expected, no LAT expression was seen in dLAT2903-infected cells. The membranes were reprobed with the host house keeping gene, GAPDH, to ensure equal loading of RNA. To our surprise, the GAPDH transcript was reduced in HSV-1–infected cells compared to mock-infected cells, although the message for GAPDH was not obviously different among the cells infected with the three viruses (Figure 2C, *middle panel*). Ethidium bromide staining of the gel is shown (Figure 2C, *lower panel*) to ensure the quality and the quantity of loaded RNA.

Replication of ΔUOL in rabbit eyes

Rabbits were infected with 2×10^5 PFU/eye. Tear films were collected from five rabbit eyes on days 3, 5, 7, and 10 p.i. (post infection). Figure 3A, B (replication) show the amount of virus detected in the eye swabs at each time point. Replication of ΔUOL appeared to be similar to that of dLAT2903 and wild type in rabbit eyes in both experiments. In addition, the peak virus titers (day 7 p.i. for experiment 1 and

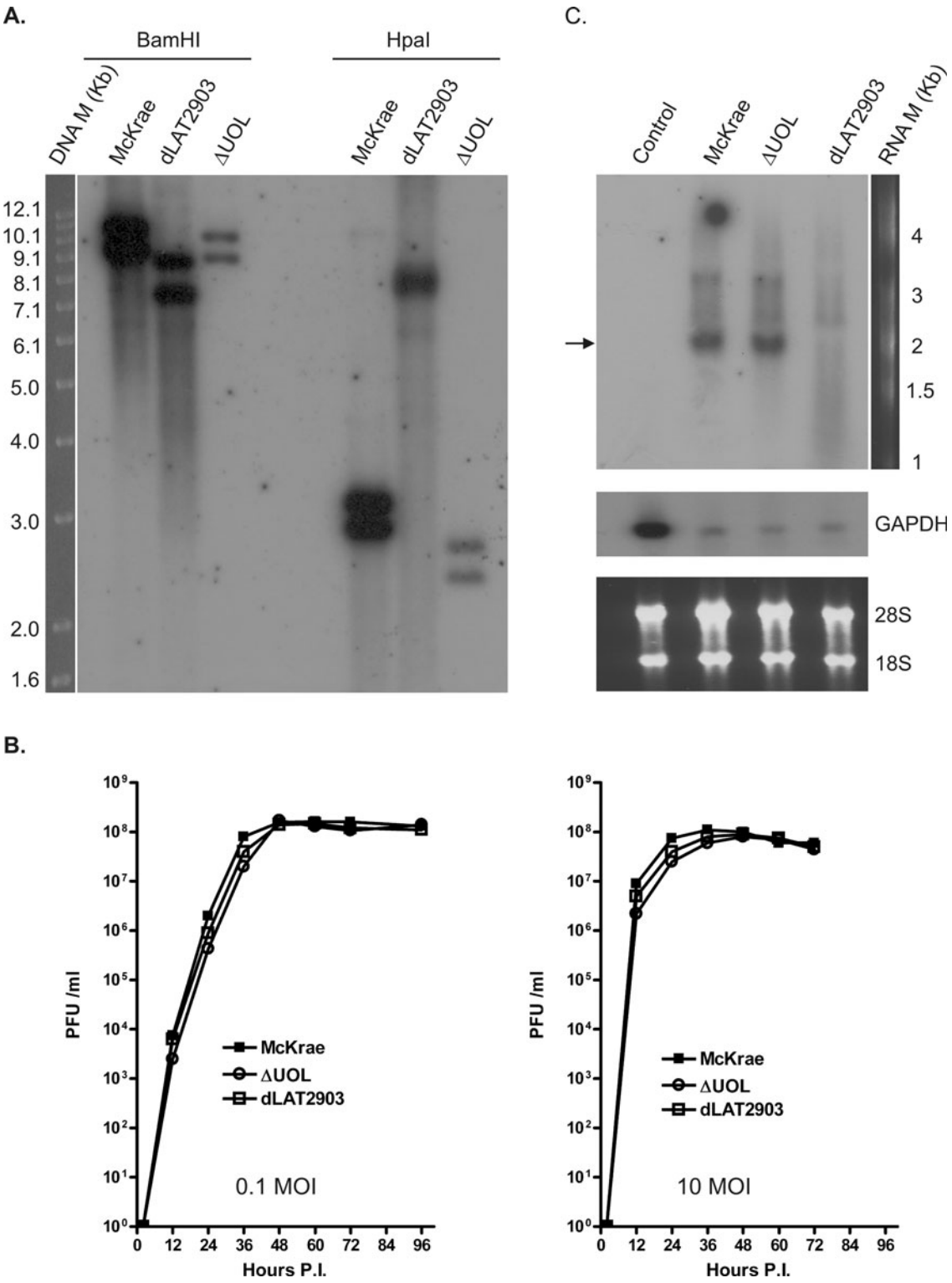

Figure 2 Characterization of ΔUOL in tissue culture. (**A**) Southern analysis of ΔUOL. Purified viral genome was digested with BamHI or HpaI, separated in agarose gel, transferred onto membrane, and hybridized with specific probe as described in Materials and Methods. (**B**) Replication of ΔUOL in tissue culture. CV-1 cell monolayers were infected with ΔUOL, dLAT2903, or McKrae, at an MOI of 0.1 (*left panel*) or 10 (*right panel*), and individual plates were harvested at the indicated times and total virus yield was determined as described in Materials and Methods. (**C**) Northern analysis of 2-kb LAT in ΔUOL. CV-1 cells were infected with indicated viruses. Northern blot was performed as described in Materials and Methods. *Upper panel*, probed with LAT-specific probe, arrow indicates the major 2-kb LAT; *middle panel*, the membrane was reprobed with GAPDH; *lower panel*, ethidium bromide staining the gel. Wild-type McKrae, parental strain of ΔUOL, and dLAT2903; ΔUOL, UOL deletion mutant virus; dLAT2903, a LAT-null mutant virus. GAPDH, host reference gene. 18S and 28S, host ribosomal RNA; DNA M (kb), 1 Kb DNA ladder; RNA M (kb), RNA Ladder.

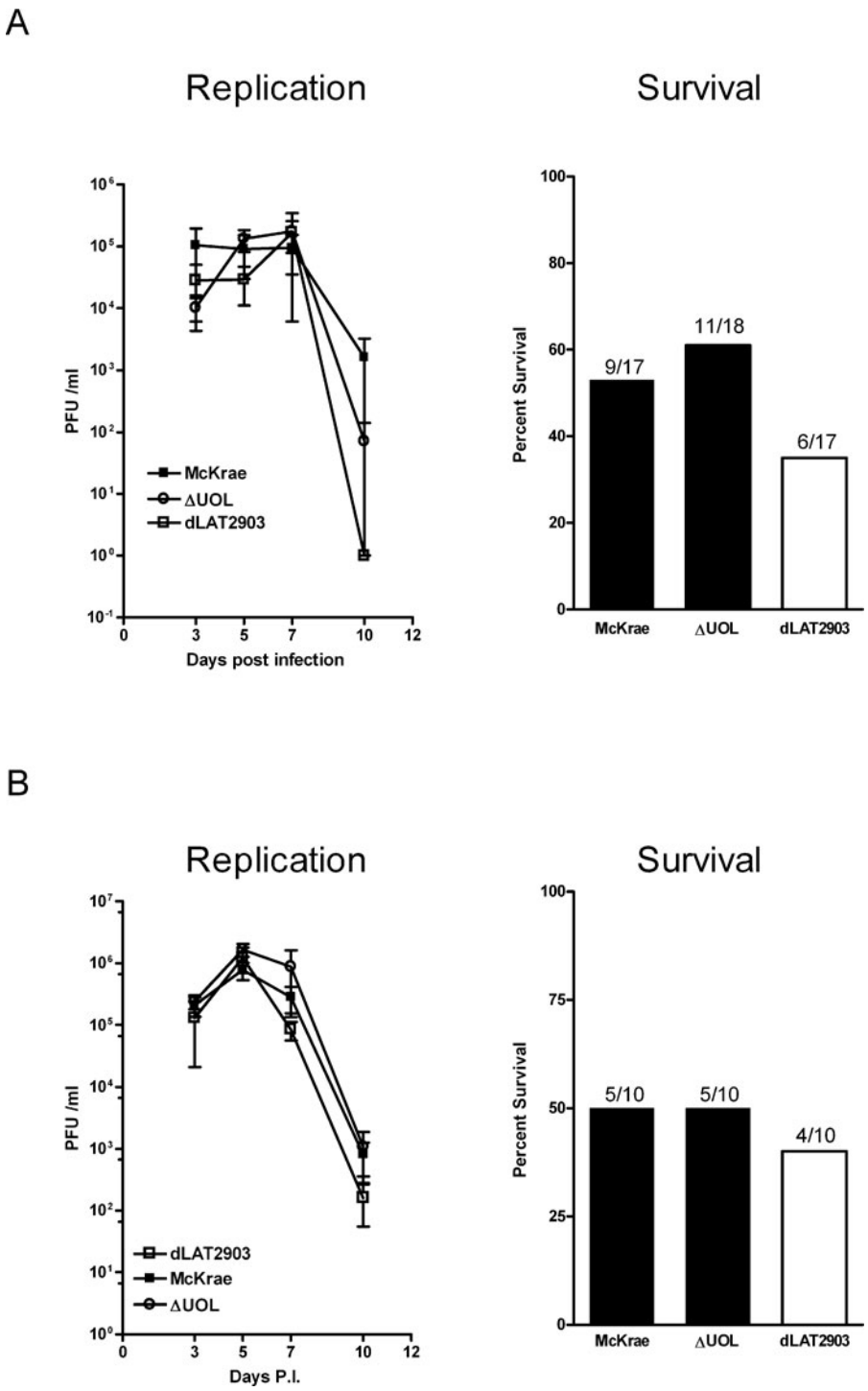

Figure 3 ΔUOL in infected rabbits. Ocular infection of rabbits, collection of eye swabs, titration of virus, and monitoring the survival of rabbits were described in Materials and Methods. Two individual experiments were performed. (**A**) Experiment 1. (**B**) Experiment 2. Wild-type McKrae, parental strain of ΔUOL, and dLAT2903; ΔUOL, UOL deletion mutant virus; dLAT2903, a LAT-null mutant virus.

day 5 for experiment 2) appeared very similar for all three viruses (Figure 3, A and B, replication).

Survival following ocular ΔUOL infection

Survival was quantitated 21 days post infection in the rabbits (Figure 3, A and B, and Table 1, Survival). Although not statistically significant, there was a suggestion in these experiments for increased survival in the rabbits infected with ΔUOL (57%, total from experiments 1 and 2; Table 1, column 3) compared to dLAT2903 (37%; Table 1, column 3) and wild-type McKrae (52%; Table 1). Thus, it was of interest to determine if significant differences might be seen with larger numbers of animals. Due

Table 1 Summary of spontaneous reactivation in survival rabbits[a]

	Virus	Survival (%)[b]	Positive eye swab culture (%)[c]	P value[d] Versus McKrae	P value[d] Versus ΔUOL
Experiment 1	McKrae	9/17 (53%)	50/468 (10.7%)		0.6
	ΔUOL	11/18 (61%)	51/572 (8.9%)	0.6	
	dLAT2903	6/17 (35%)	8/312 (2.6%)	0.0001	0.0014
Experiment 1	McKrae	5/10 (50%)	31/260 (12%)		0.9
	ΔUOL	5/10 (50%)	35/260 (13%)	0.9	
	dLAT2903	4/10 (40%)	6/208 (2.9%)	0.0015	0.0003
Total	McKrae	14/27 (52%)	81/728 (11.1%)		0.88
	ΔUOL	16/28 (57%)	86/832 (10.3%)	0.88	
	dLAT2903	10/27 (37%)	14/520 (2.7%)	<0.0001	<0.0001

[a]Rabbits were ocularly infected with McKrae, ΔUOL, or dLAT203 at 2×10^5 PFU/eye as described in Materials and Methods. Rabbits surviving past 28 days post infection are assumed to establish latency. Beginning 31 days after infection, eye swabs were collected daily for 26 days.
[b]Rabbits were ocularly infected as described in Materials and Methods. Survival was determined on day 21 post infection as the number of surviving animals/total rabbits infected with each virus.
[c]Virus-positive tear cultures per total number of cultures. The number of cultures is equal to the number of eyes times 26 days (the length of the study period).
[d]Fisher exact test. $P < .05$ indicates statistical significance.

to difficulties with using large numbers of rabbits, we decided to examine survival in mice (see survival in mice below).

Spontaneous reactivation of ΔUOL in latently infected rabbits

Beginning at 31 days post infection, all eyes from the surviving rabbits in experiments 1 and 2 were swabbed once a day for 26 days to collect tear films for the presence of reactivated infectious virus as described in Materials and Methods. The cumulative number of virus-positive tear film cultures is shown in Figure 4A and B. In both experiments, ΔUOL reactivated as efficiently as wild-type McKrae. Both ΔUOL and McKrae reactivated far more efficiently than that of dLAT2903, which has a low reactivation phenotype (Perng *et al*, 1994a).

The cumulative data for positive cultures versus total cultures in experiment 1 (Table 1, experiment 1, column 4) indicated that 8.9% of the tear film cultures from rabbits latently infected with ΔUOL contained spontaneously reactivated virus compared to 2.6% of the tears from eyes infected with dLAT2903. In contrast, 10.7% of tears from eyes infected with McKrae contained spontaneously reactivated virus. The rate of spontaneous reactivation as judged by the positive cultures versus total cultures between ΔUOL and McKrae was similar to each other (Table 1, experiment 1, column 5, $P = .6$), whereas either ΔUOL or McKrae virus spontaneous reactivation was

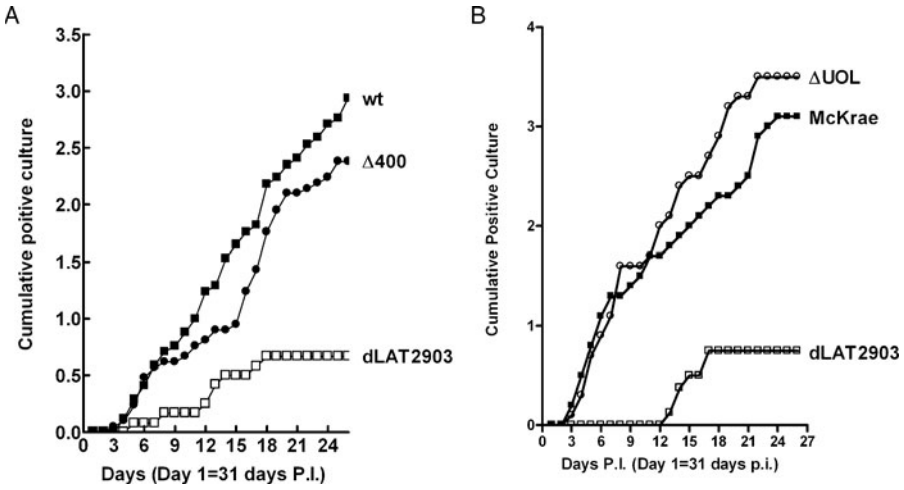

Figure 4 Spontaneous reactivation of ΔUOL. Ocular infection of rabbits and collection of eye swabs were described in Materials and Methods. Beginning 31 days after infection, eye swabs were collected daily from the surviving rabbits, placed on the RS indicator cells for the presence of reactivated infectious virus. Two independent experiments were performed. (**A**) Experiment 1. (**B**) Experiment 2. Wild-type McKrae, parental strain of ΔUOL, and dLAT2903; ΔUOL, UOL deletion mutant virus; dLAT2903, a LAT-null mutant virus.

significantly higher than dLAT2903 (Table 1, experiment 1, column 5, $P = .0014$ and .0001, respectively).

In experiment 2, the cumulative spontaneous reactivation in rabbits latently infected with ΔUOL also appeared to be similar to that of McKrae (Figure 4B). Again, both viruses appeared to reactivate more efficiently than dLAT2903. The cumulative number of positive cultures versus total cultures (Table 1, experiment 2, column 4) indicated that 13% of the ΔUOL and 12% of McKrae tear film cultures were positive, whereas only 2.9% of the dLAT2903 cultures were positive. The increment in ΔUOL spontaneous reactivation was not significantly different from McKrae (Table 1, experiment 2, column 5, $P = .9$) but was significantly different from dLAT2903 (Table 1, experiment 2, column 5, $P = .0003$). The spontaneous reactivation in rabbits latently infected with McKrae was significantly higher than that of dLAT2903 (Table 1, experiment 2, column 5, $P = .0015$).

The totals of the two experiments were analyzed for spontaneous reactivation. The spontaneous reactivation rate of ΔUOL was similar to that of McKrae (Table 1, total, column 5, $P = .88$). In contrast, the spontaneous reactivation of either ΔUOL or McKrae compared to dLAT2903 was highly significant (Table 1, total, column 5, $P < .0001$). Thus, deletion of the 437 nts in UOL did not impair spontaneous reactivation.

Replication of ΔUOL in mouse eyes, TGs, and brains
Mice were infected with 2×10^5 PFU/eye and replication in eyes was examined. Figure 5A shows the results of the experiments. Ocular replication of ΔUOL and ΔBsa, a deletion of the 3′ end of UOL on an HSV1 strain 17syn+ background rather than McKrae (Zhu *et al*, 1999), was similar to that of wild type, dLAT2903, or ΔBsaR, a marker rescue of ΔBsa (Zhu *et al*, 1999). Thus, alterations in the UOL gene did not appear to alter ocular replication in mouse eyes.

For analysis of replication in mouse TG, 5 mice were ocularly infected per group for each time point shown (Figure 5B) and the amount of infectious virus was determined individually for each TG at each time point. There was no difference among the viruses at any time point. The amount of virus in the brains of the same mice was similarly determined (Figure 5C). The amount of virus on peak days (day 7 p.i.) in the brains of mice infected with ΔUOL or ΔBsa was similar to that of wild type, dLAT2903, or ΔBsaR. Thus, alterations in the UOL gene also did not appear to have a significant effect virus replication in mice TG or brains.

Virulence of ΔUOL in mice
Groups of 45 mice were infected with 2×10^5 PFU/eye of ΔUOL, ΔBsa, ΔBsaR, dLAT2903, or wild-type McKrae. Virulence (death due to encephalitis) was determined by survival on day 21 after ocular infection. The results are plotted as the percentage of mice that survived (Figure 6A). The P values were determined from survival curves (log rank test) using GraphPad Prism 4.0 for Windows (GraphPad Software). The survival rate of mice infected with ΔUOL was approximately 70% (31/45), which was very similar to mice infected with ΔBsa, 80% (36/45) survival. In contrast, survival rates of mice infected with ΔBsaR (23/45), dLAT2903 (23/45), or McKrae (20/45) were approximately 51%, 51%, and 44%, respectively. In comparing the survival curves, mice infected with ΔUOL had similar survival to ΔBsa ($P = 0.26$) but were less virulent than McKrae ($P = .009$), dLAT2903 ($P = .04$), or ΔBsaR ($P = .043$). In addition, ΔBsa was less virulent than wild-type McKrae ($P = .0004$), dLAT2903 ($P = .0025$), or ΔBsaR ($P = .0028$). This is consistent with Zhu *et al* (1999). Thus, as judged by survival of mice following ocular HSV-1 challenge, ΔUOL was less virulent than the original wild-type parental virus (McKrae) or dLAT2903.

Trigeminal ganglia explant reactivation of ΔUOL
The mice that survived to the end of the experiment (Figure 6A) were used to examine the reactivation phenotype of ΔUOL in mice (Figure 6B). Because more mice survived in the ΔUOL- and ΔBsa-infected groups than in the groups infected with dLAT2903, McKrae, or ΔBsaR, 15 mice per group were randomly selected from each infected group so that the number of TG (i.e., 30) in each mouse group would be the same. Thus, any apparent differences in explant-induced reactivation among the groups should not be due to unequal statistical power. On day 31 p.i., the mice were euthanized, and individual TG was removed and cultured in tissue culture media. Aliquots of medium were removed from each culture daily for up to 18 days and plated on indicator cells (RS cells) to look for the appearance of reactivated virus. Because the medium from the explanted TG cultures were plated daily, the time at which reactivated virus first appeared in the explanted TG cultures could be determined. The results were plotted as the cumulative percentage of TG that reactivated (Figure 6B). No significant difference in reactivation was detected among wild-type McKrae, ΔUOL, ΔBsa, or ΔBsaR ($P > .1$, log rank test, Figure 6B), whereas as expected reactivation of dLAT2903 was significantly lower. Thus, consistent with the rabbit results shown in Figure 4, alteration of the UOL gene did not appear to have an effect on the reactivation phenotype in mice.

Discussion

Following primary ocular infection, HSV-1 travels up sensory nerves and establish latent infection in neurons of the trigeminal ganglia via mechanisms that remain to be fully defined. The latent virus can reactivate sporadically throughout the life of the individual and produce recurrent disease. The host factors contributing to and/or effecting latency, establishment,

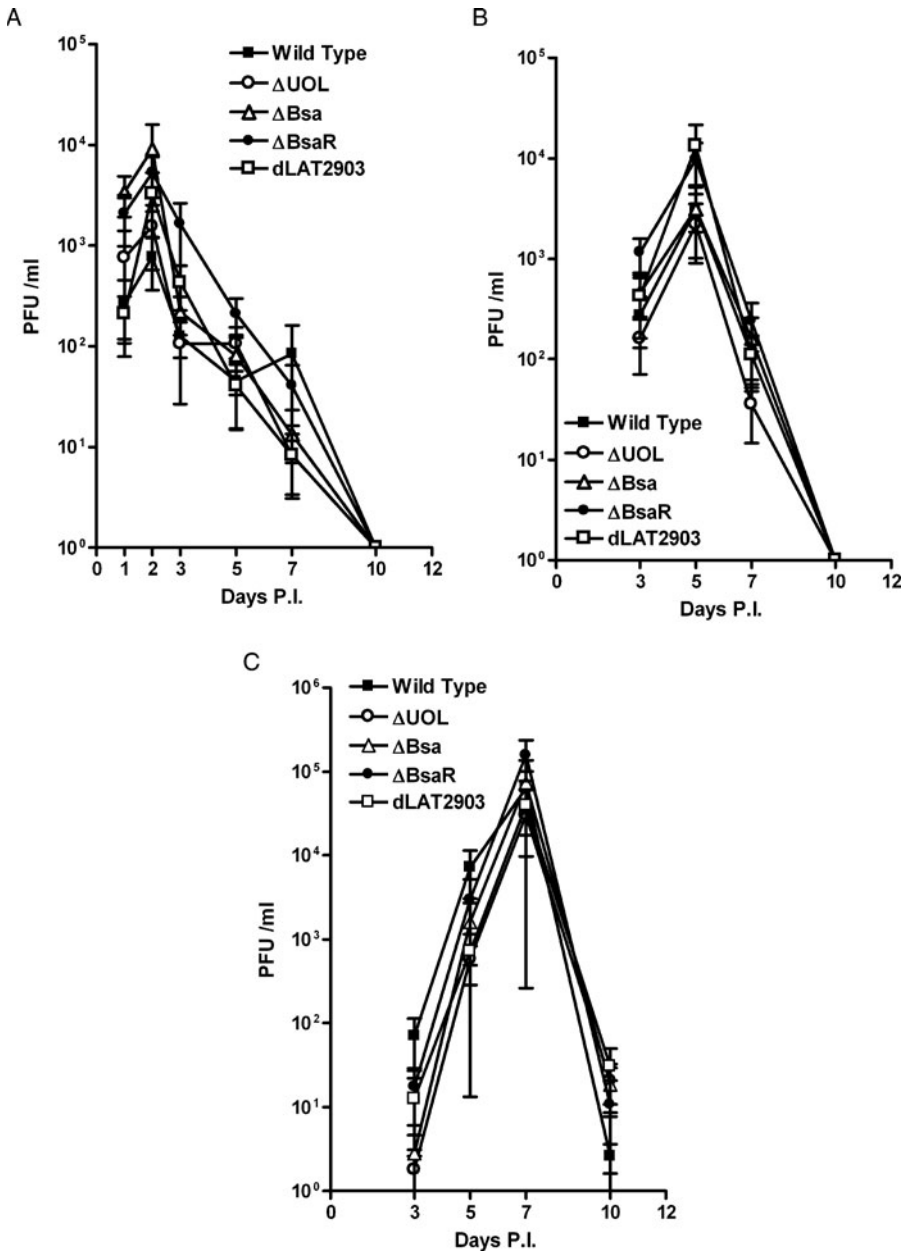

Figure 5 Replication of ΔUOL in mice. Ocular infection of mice, eye swabs taken, collection of Tg, or brains, and viral titration were described in Materials and Methods. (**A**) Replication of ΔUOL in eyes. (**B**) Replication of ΔUOL in Tg. (**C**) Replication of ΔUOL in brains. Wild-type McKrae, parental strain of ΔUOL, and dLAT2903; ΔUOL, UOL deletion mutant virus; dLAT2903, a LAT-null mutant virus; ΔBsa, a 3′ end UOL deletion mutant in 17syn+ strain; ΔBsaR, a rescue of ΔBsa.

maintenance, and reactivation remain virtually unknown after decades of investigation. Thus, altering viral genes involved in the HSV-1 latency-reactivation cycle is currently the main approach for understanding viral pathogenesis and latency.

Because LAT is the only viral gene product readily detected during latent infection, it has been the major subject of investigation. The LAT locus (the region in and around the 5′ end of LAT) plays an important role in the HSV-1 latency-reactivation cycle (review in Jones, 2003). However, differences have arisen between different HSV-1 strains and different animal models (Block *et al*, 1993; Devi-Rao *et al*, 1991; Hill *et al*, 1990; Loutsch *et al*, 1999; Perng *et al*, 2001b). Thus, even though a deletion mutant in the region immediately upstream of the LAT promoter has been reported using strain 17syn+ the mouse model to examine explant-induced reactivation (Maggioncalda *et al*, 1996; Zhu *et al*, 1999), it was useful to examine a similar mutant in a different strain background, such as McKrae, and examine its effect on spontaneous reactivation in the rabbit model.

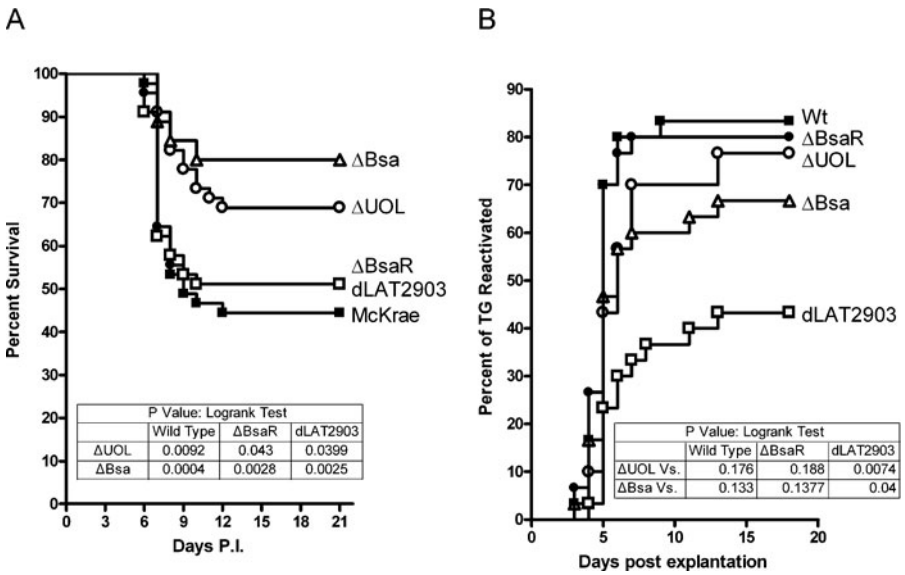

Figure 6 Virulence and reactivation of ΔUOL. Ocular infection of mice, monitoring the mouse survival, and TG explant reactivation assays were described in Materials and Methods. (**A**) Survival. Infected mice were monitored daily and the surviving animals were recorded up to 21 days after infection. The survival curve was plotted with GraphPad 4.0 software (GraphPad, San Diego, CA). (**B**) Tg explant cocultivation assays. The procedures were described in Materials and Methods. The cumulative positive cultures were plotted as an increment of survival curve using GraphPad 4.0 software as a percentage of TGs that were positive for reactivated virus over the total TG. The statistics were analyzed with logrank test, $P < .05$ is considered significant. Wild-type McKrae, parental strain of ΔUOL, and dLAT2903; ΔUOL, UOL deletion mutant virus; dLAT2903, a LAT-null mutant virus; ΔBsa, a 3′ end UOL deletion mutant in 17syn+ strain; ΔBsaR, a rescue of ΔBsa.

Detection of transcripts in the HSV-1 17syn+ genome upstream of LAT has been reported by several groups (Ben-Hur *et al*, 1989; Singh and Wagner, 1993; Zhu *et al*, 1999). Singh and Wagner detected 5′ coterminal 1.1/0.9- and 1.8-kb transcripts. Mapping studies designed to knock out only the 1.1/0.9-kb transcript did not have an effect on induced TG explant reactivation in latently infected mice (Maggioncalda *et al*, 1996). Zhu et al. set out to reexamine the transcripts in this region of the HSV-1 genome and discovered a novel 0.7-kb transcript (Zhu *et al*, 1999). Knocking out the 3′ end of this transcript also did not appear to have an effect on induced explant reactivation in mice (Zhu *et al*, 1999). Whether the 0.7-kb transcript is the 1.1/0.9-kb transcript reported by Singh and Wagner (1993) is unclear. In addition, we previously cloned and characterized a novel protein, UOL, within the LAT promoter region of McKrae (Naito *et al*, 2005). The McKrae UOL gene appears to correspond to the 17syn+ 0.7-kb RNA reported by Zhu *et al* (1999).

We constructed an HSV-1 strain McKrae mutant, ΔUOL, in which a 437-nt segment is deleted. The deletion knocks out most of the UOL open reading frame and also a portion of the presumed LAT promoter regulatory region, although the core LAT promoter and TATA box are unchanged. As found with the similar previously reported 17ΔS/N mutant (Maggioncalda *et al*, 1996), ΔUOL appeared similar to its parental wild-type virus as regards LAT transcription, replication in tissue culture, replication in mouse eyes, TG, and brain, and explant TG reactivation in the mouse. The spontaneous reactivation phenotype of ΔUOL in rabbits was also similar to its parental wild-type McKrae. Thus, the UOL gene did not appear to be critical for the HSV-1 reactivation phenotype.

Although virulence (death of animals due to encephalitis) was not reported for the 17ΔS/N mutant (Maggioncalda *et al*, 1996), we observed a trend towards reduced virulence of ΔUOL in rabbits. In addition, although the peak titers of ΔUOL and ΔBsa in mouse brains were similar to wild type, on day 3 p.i. it appeared that both mutants had reduced titers compared to wild type. Using a larger number of mice, we found that significantly more animals infected with ΔUOL or ΔBsa survived compared to wild type. Thus, UOL may play a role in neurovirulence following ocular infection. The lack of statistical significant in rabbits may have been a result of less statistical power due to the use of fewer animals/group compared to the mouse experiment. Alternatively it may represent an asymmetric effect of the UOL mutant on virulence in mice compared to rabbits, as we have previously reported for other mutants near the 5′ end of LAT (Perng *et al*, 2001a).

The asymmetric virulence results in rabbits versus mice with ΔUOL were unexpected. Nonetheless, mutations that alter the virulence of HSV-1 in mice but not rabbits have been reported (Zhu *et al*, 1999). Thus, although the viral genome from input (before inoculation) or from reactivated (recovered

from reactivation) viruses appeared to be identical, analyzed by restriction enzymes digestion and Southern analysis, we do not rule out the possibility that a minor mutation along the ΔUOL genome may exist.

Materials and methods

Virus and cells
The parental virus, HSV-1 McKrae, and all mutants were triple plaque purified and passed only one or two times in rabbit skin (RS) cells prior to use. ΔBsa, a deletion of the 3′ end of UOL in 17syn+ strain, and its rescue, ΔBsaR, have been described (Naito *et al*, 2005; Zhu *et al*, 1999). RS and CV-1 cells were grown in Eagle minimal essential medium supplemented with 5% fetal calf serum.

Construction of UOL deletion virus
The UOL deletion virus was constructed according to our previously published protocol (Perng *et al*, 1994b). Briefly, the HSV viral genome was purified and digested with HpaI, with the digested DNA separated on agarose gel. The desired DNA fragment, HpaI P (Figure 1), was isolated and cloned into a PUC19 plasmid. The resulting plasmid, HpaI-PUC19, was cotransfected with infectious HSV-1 DNA onto RS cells. A serial dilution of the cotransfection mixtures was made and plated onto a RS cell monolayer and overlaid with agarose. Individual plaques were picked and grown in a RS cell monolayer, viral DNA was isolated, digested with the appropriate restriction enzyme, separated on agarose gel, transferred onto a membrane, and probed with a radiolabeled DNA fragment corresponding to the region of UOL. A plaque containing the desired deletion (UOL) was plaque purified further for three rounds. The resulting virus was designated ΔUOL. The schematic description of the relative location of UOL to that of LAT promoter is shown in Figure 1.

Replication of ΔUOL in CV-1 cells
Non-neuronal derived CV-1 fibroblasts were used in this experiment. Monolayer of CV-1 cells seeded in 60-mm tissue culture plates were infected with wild type (McKrae), ΔUOL, or dLAT2903, at an MOI of 0.1 or 10. Plates were harvested at various time points, freeze-thawed, and total virus was determined by standard plaque assays on RS cells as previously described (Perng *et al*, 1994a).

RNA isolation from infected cells
CV-1 cells were infected with McKrae, dLAT2903, or ΔUOL virus at 5 PFU/cell. Total RNA was isolated from infected and mock-infected cells 24 h post infection by the Trizol buffer method. For each sample, 5 ml of Trizol RNA isolation reagent supplemented with 7 μl of β-mercaptoethanol was added to the infected cells to lyse the cells. All the materials were then transferred to a 5-ml snap cap tube. One-tenth volume of chloroform was added to the tube. The entire mix was vortexed for at least 40 s or until an emulsification was formed. The tube was kept in ice for 30 min, spun at 2500 rpm at 4°C for 20 min, and the upper layer containing the RNA was carefully pipetted to a new snap cap tube. RNA was precipitated with at least two volumes of isopropanol, mixed gently, and stored at −20°C for at least 24 h. The RNA was concentrated by spinning the tube at 8000 rpm for 20 min at 4°C. The supernatant was decanted and the RNA pellet was then washed by resuspension in 5 ml of RNA grade 70% ethanol. The RNA was then respun at the same conditions and following decanting of the waste Ethanol was vacuum dried. Qiagen RNeasy isolation kit was used for further purification of RNA. The concentration of purified total RNA was measured by spectroscopy. The purified RNA was then kept at −80°C until use.

Separation of RNA and Northern blot
Ten micrograms of total RNA was run on a RNA agarose gel according to the standard protocols established in the lab. At the end of run, the RNA was transferred onto a 0.22-μm Nylon membrane by capillary method overnight. The Nylon membrane was dried and UV cross-linked for 2 min, prehybridized with blocking solution for at least 2 h, and probed with ^{32}P-labeled HSV-1 DNA fragments (LAT-specific and GAPDH-specific) in blocking solution overnight at 68°C. The hybridized membrane was washed several times with buffer solution under stringent conditions. The membrane was then exposed to an x-ray film for overnight to several days, and the film was developed using the AFP Imacine automatic developer.

Rabbits
Eight- to 10-week-old New Zealand white male rabbits (Irish Farms) were used. Rabbits were treated in accordance with ARVO (Association for Research in Vision and Ophthalmology), AALAC (American Association for Laboratory Animal Care), and National Institute of Health guidelines. Rabbits were bilaterally infected without scarification or anesthesia by placing, as eye drops, 2×10^5 PFU of virus into the conjunctiva cul-de-sac, closing the eyes, and rubbing the lid gently against the eye for 30 s as previously described (Perng *et al*, 1994a). Analysis of virus replication in eyes was done as previously described (Perng *et al*, 1994a). Virulence (death due to encephalitis) was determined by survival at 21 days after infection as previously described (Perng *et al*, 1999, 2001a).

Spontaneous reactivation
Beginning on day 31 post infection, tear film specimens were collected daily from each rabbit eye for 26 days as previously described (Perng *et al*, 1994a, 1996a, 1996b), using a nylon tipped swab. The swab was then placed in 0.5 ml tissue culture medium, squeezed, and the inoculated medium was used to

infect RS cell monolayers. These cell monolayers were observed in a masked fashion by phase light microscopy for up to 5 days for HSV-1 cytopathic effects (CPE). All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. DNA was purified from positive cultures and analyzed by restriction enzyme digestion and Southern blot analysis to confirm that the CPE was due to reactivated HSV-1 and that the reactivated virus was identical to the input virus.

Mice
Eight- to 10-week-old female Swiss-Webster mice were used. Mice were ocularly infected with 2×10^5 PFU/eye without corneal scarification as previously described (Mott *et al*, 2003; Perng *et al*, 2001b). Mice corneas were scarified with no. 25 gauge needle prior to ΔBsa or ΔBsaR virus infection. Tear films were collected at various days after infection from one eye per animal. The amount of virus in each tear film was determined by standard plaque assays on RS cells. Virulence (death due encephalitis) was determined by survival on day 21 after ocular infection as previously described (Mott *et al*, 2003; Perng *et al*, 2001b).

Virus replication in mouse trigeminal ganglia and brains
Mice were infected as above and euthanized at various times post infection. The brain and TG were removed and individually homogenized, debris pelleted by low speed centrifugation, and the amount of infectious virus in the supernatant was determined by standard plaque assays on RS cells as previously described (Perng *et al*, 2001a).

Mouse explant cultivation reactivation assay
Mice were sacrificed at 30 days post infection and individual TG (two per mouse) was cultured in tissue culture media. Aliquots of media were removed from each culture daily for up to 18 days and plated on RS cells to monitor for the presence of reactivated virus as previously described (Perng *et al*, 2001b).

Statistical analysis
Statistical analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). Results were considered statistically significant when the P value was $<.05$.

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