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# The herpes simplex virus type 1 latency associated transcript locus is required for the maintenance of reactivation competent latent infections

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**Abstract** Herpes simplex virus (HSV) establishes latent infections in sensory neurons from which it can periodically reactivate and cause recurrent disease and transmission to new hosts. Little is known about the virally encoded mechanisms that influence the maintenance of HSV latent infectious and modulate the frequency of virus reactivation from the latent state. Here, we report that the latency associated transcript locus of HSV-1 is required for long-term maintenance of reactivation competent latent infections.

**Keywords** Herpes simplex virus type 1 · Latent infections · Innervating sensory neurons · Latency associated transcript · Maintenance of latency · Reactivation · Mouse model

Herpes simplex virus type (HSV) infects and replicates at the body surface and establishes latent infections in innervating sensory neurons. Stressful stimuli can cause reactivation of the latent virus and recurrent disease, which facilitates infection of new hosts (Roizman et al. 2007). While most reactivation events are asymptomatic, recurrence in the eye is a leading cause of blindness (Pepose et al. 2006; Toma et al. 2008), and reactivation of HSV in the genital tract is associated with a 3-fold higher transmission rate of HIV (Glynn et al. 2009; Fuchs et al. 2010). In addition, rare but serious sequelae including fatal encephalitis can occur (Stone and Hawkins 2007). In experimental models, the frequency of reactivation is directly correlated with the level of latent infections (Sawtell 1998; Sawtell et al. 1998; Sawtell 2003), but factors that influence the establishment and maintenance of latent infections are not well understood.

In our mouse model, about 6,000 neurons per TG are latently infected as defined by the presence of the viral genome (Sawtell 1997; Thompson and Sawtell 1997; Sawtell et al. 1998; Thompson and Sawtell 2001). Spontaneous reactivation occurs in one neuron out of 20 TG or about 1/120,000 latently infected neurons per day (Sawtell 1998). Stressful stimuli including hyperthermic stress (HS) increase the reactivation frequency about 50-fold to ~1/2,500 (Sawtell 1998; Sawtell et al. 1998; Thompson et al. 2009). Clearly, the maintenance of latent infections is tightly controlled, but little is known about viral functions that maintain the latent state.

The latency associated transcript (LAT) locus has been extensively investigated and shown to express several unusual forms of non-coding RNAs during latency including a primary transcript, stable introns spliced from this transcript, small RNAs, and microRNAs, one of which is upstream of the LAT promoter and encoded on the opposite strand (Rock et al. 1987; Stevens et al. 1987; Wechsler et al. 1988; Farrell et al. 1991; Peng et al. 2008; Umbach et al. 2008). In addition, some evidence of viral proteins expressed during latency (most from the LAT region) has been reported (Green et al. 1981; Doerig et al. 1991; Thomas et al. 1999; Perng et al. 2002; Thomas et al. 2002; Henderson et al. 2009; Jaber et al. 2009), but the functions of these proteins are not known.

We and others have shown that HSV-1 LAT mutants can establish latent infections and reactivate from them (Sedarati et al. 1989; Sawtell and Thompson 1992a, b; Bloom et al. 1994; Perng et al. 1994; Thompson and Sawtell 1997, 2001). Analysis at the single neuron level reveals that significantly fewer latent infections are established by LAT null

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mutants demonstrating a role for this locus during the establishment of latency (Sawtell and Thompson 1992a, b; Thompson and Sawtell 1997, 2001; Perng et al. 2000). In the mouse model, this is likely to be due to a function that serves to suppress lytic viral protein production in TG neurons (Sawtell and Thompson 1992a, b; Garber et al. 1997; Thompson and Sawtell 1997). In the absence of the LAT locus, significantly more neurons expressing the LAT promoter enter into lytic infection and fewer neurons survive (Sawtell and Thompson 1992a, b; Thompson and Sawtell 1997, 2001).

The LAT locus is expressed in a subset of neurons during latency (Deatly et al. 1987; Rock et al. 1987; Stevens et al. 1987; Arthur et al. 1993). We hypothesize that regulators encoded in the LAT locus provide function(s) that repress entry into the lytic cycle, and this in turn serves to moderate the frequency of reactivation and maintains the pool of latently infected neurons through time (Sawtell and Thompson 1992a, b; Garber et al. 1997; Thompson and Sawtell 1997, 2001; Kang et al. 2003). For example, there is a post transcriptional constraint on the production of ICP0 mediated by the LAT locus during in vivo reactivation (Thompson et al. 2003). In this way, the frequency of reactivation and or the number of neurons exiting latency per reactivation event could be limited. This would be important to minimize loss from the pool of latently infected neurons, which, in turn, would increase the probability of transmission to new hosts throughout the life of the infected individual. If this hypothesis is true, then reactivation competent latent infections established by LAT null viruses should be more prone to reactivate than those established by viruses that have an intact LAT locus.

## Results

In previous experiments with 17syn+, we found that the frequency of reactivation is not significantly reduced during long term latency (spanning >250 days) (Sawtell 2003). In addition, preliminary experiments revealed that when mice latently infected with 17syn+ were induced to reactivate multiple times over a 5-month period using hyperthermic stress (HS), a physiologically relevant stress that induces reactivation from latency in vivo (Sawtell and Thompson 1992a, b), the final reactivation frequency was not different from that after a single HS (66% vs 68%, respectively). This information and experimental setup was used to directly test whether reactivation competent latent infections established by the LAT null mutant 17AH would be depleted over time. The construction of 17AH has been described previously including the characterization of multiple independently derived isolates of 17AH and its genomically restored counterpart, 17AHR. The replication of 17AH and 17AHR are equivalent in vitro and in vivo replication of 17AH and 17AHR in eyes, and TGs are not different (Thompson and Sawtell 1997; Thompson and Sawtell 2001). The genomic structure of 17AH and location of the latency associated riboregulators are shown in Fig. 1. The ~1.9-kb deletion in 17AH eliminates the LAT promoter and deletes exon one of the LAT primary transcript past the splice donor site utilized by the stable LAT introns (Thompson and Sawtell 1997). The deletion does not eliminate the putative promoter and AL3 ORF located on the strand opposite of LAT (Henderson et al. 2009) but might affect the stability of this potential messenger RNA. No detectable LAT-related RNA is expressed in

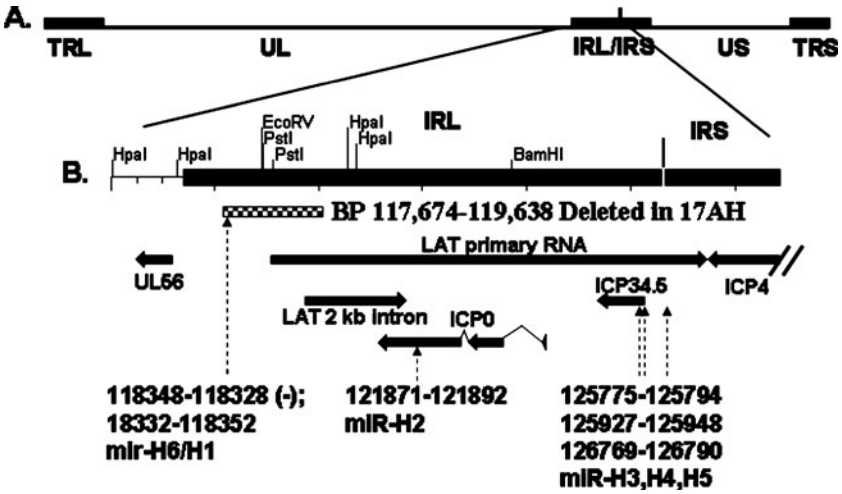


**Fig. 1** The genomic structure of the latency associated riboregulators null mutant employed in these studies. **a** A schematic of the prototypical arrangement of the HSC-1 genome is shown schematically with the unique long (UL) and unique short (US) labeled. *Heavy black lines* denote the terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS) separated from IRL with a *vertical line*, and terminal repeat short (TRS) indicated. **b** A region showing the IRL is enlarged. The location of the ~2 kb deletion present in mutant 17AH is indicated by a *stippled bar* with the base pairs deleted indicated. The same region is deleted in the TRL (not shown). The location of well characterized viral ORFs and a subset of putative riboregulators are indicated

neurons in vivo by this mutant (Thompson and Sawtell 1997), and the latency related microRNA miR-H6 that resides upstream of the LAT basal promoter (Umbach et al. 2008) is also deleted as shown (Fig. 1).

Groups of outbred 5–6-week-old Swiss Webster male mice were infected with $1\times10^5$ pfu of the mutant 17AH and $3\times10^4$ pfu of the genomically restored isolate 17AHR. The amount of 17AHR employed to infect the mice was adjusted so that equivalent levels of latency were obtained in both groups as previously detailed (Sawtell et al. 2006; Thompson and Sawtell 2006; Thompson et al. 2009). The mice were maintained for 30 days post-inoculation at which time the 17AH and 17AHR infected mice were each divided into two groups. One half of the latently infected mice were maintained an additional 294 days. Additional mice from these same groups were subjected to the hyperthemic stress procedure an average of 2.5 times per week during 280 days after the initial 30-day period. After week 40, the mice receiving the multiple HS treatments were rested for 2 weeks, and then all of the mice were subjected to a final HS procedure to induce reactivation in vivo. Two measures were employed to quantify the outcome in TG at 22 hrs post HS, (1) the quantification of infectious virus and (2) quantification of the number of neurons exiting latency using whole ganglion immunohistochemistry (WGIHC) for viral proteins. With these methods, we can reliably detect a 2-fold difference in the number of neurons that enter the lytic cycle or the number of TG positive and the number of plaque-forming units they contain with 95% confidence (e.g., $p\leq0.05$ and a power of $\leq0.80$ as detailed previously (Thompson and Sawtell 1997; Sawtell 2003, 2005; Sawtell et al. 2006; Thompson et al. 2009). The results are shown in Fig. 2.

In mice receiving only a single HS at 42 weeks pi, neither the frequency (% TG reactivating) nor the number of neurons exiting latency was different between mice latently infected with 17AH or 17AHR (60% and 66% of TG positive, average=3.6 and 3.7 neurons reactivating per positive TG, $p=1.0$) (Fig. 2). In the 17AHR-infected mice that received

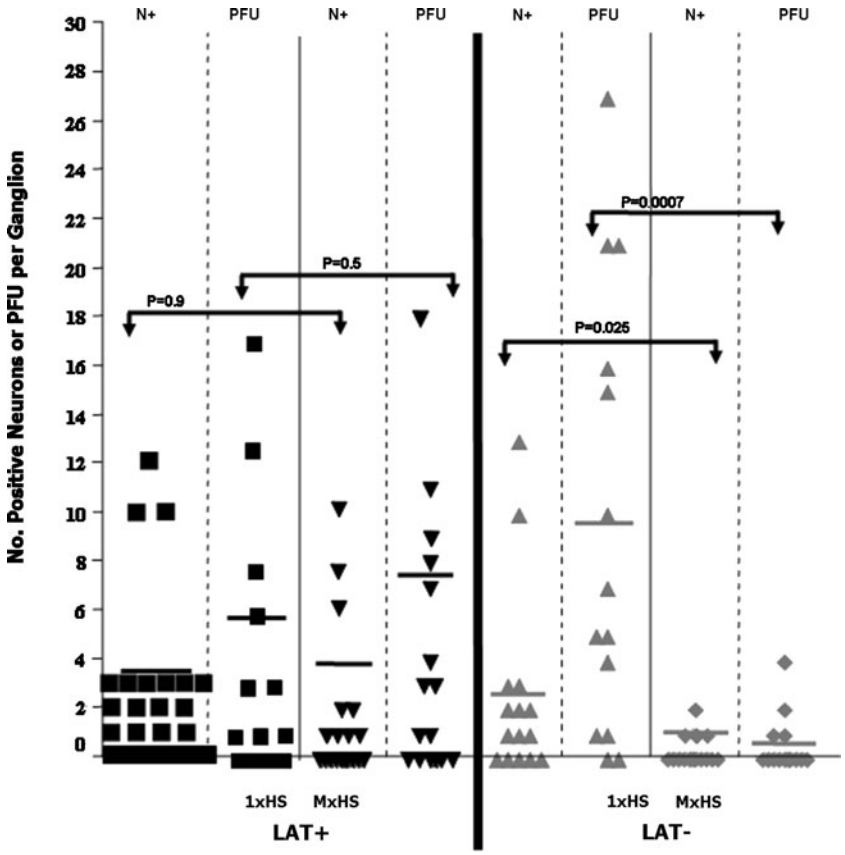


**Fig. 2** An intact LAT locus is associated with preservation of reactivation competent latent infections through time. Mice were infected with the LAT null mutant 17AH or the genomically restored wild-type derivative 17AHR as described in the text. One half of the mice were maintained for 42 weeks after latency was established, and one half of each group was subjected to HS an average of 2.5 times per week for 40 weeks, and then rested for 2 weeks. At 42 weeks post-latency, the mice received a HS procedure to induce viral reactivation in vivo. At 22 h post-HS, groups of TG were processed for the detection of viral protein positive neurons by whole ganglion immunohistochemistry or for the presence of infectious virus. Each point in the scattergram represents the results from one TG. 1xHS are the results from mice maintained for 42 weeks and subjected to a single HS treatment and MxHS are those that received multiple stresses through time. LAT+ is the 17AHR groups and LAT− is the 17AH groups. The *horizontal bars* are the mean values

either one or multiple HS treatments, no difference in either the number of neurons that exited latency, the amount of virus detected, or the frequency of detection of virus was observed ($p=0.9$ and 0.5, respectively, Mann–Whitney test). The results with 17AHR were not different than those obtained in prior experiments with the parental strain 17Syn+ in which 68% (15/22) of latently infected mice receiving a single HS treatment were positive for reactivated virus in TG vs. 66% (14/21) of those receiving multiple HS treatments through 5 months were positive and the number of plaque-forming units detected in TG was also not different ($p\geq0.7$). However, there were striking decreases in these measures in TG from mice latently infected with the LAT null virus after multiple HS treatments compared to a single treatment (Fig. 2; number of neurons positive $p=0.025$, and number of plaque-forming units detected $p=0.0007$, Mann–Whitney test). Interestingly, these results indicate that the level of spontaneous reactivation in these mice was not sufficient to reduce reactivation frequency in either LAT+ or LAT null-infected animals. Previous studies predict that approximately 30 single neuron reactivation events would occur spontaneously per mouse latently infected with wild type virus during this period (Sawtell 2003). The multiple HS applied through 40 weeks would be expected to induce about 300 reactivation events (including multiple neurons) with wild-type virus per mouse (Sawtell 2003; Sawtell and Thompson 2004; Thompson and Sawtell 2006; Thompson et al. 2009). As noted above, the mouse infections were adjusted to generate equivalent latent infections with 17AH and 17AHR. In addition, in the single HS-treated groups, both the LAT null and genomic rescue infected mice had the same reactivation frequency (as measured by infectious virus production) and equivalent frequency of entry into lytic protein production (measured by WGIHC). Because these frequencies are directly and highly correlated with the number of latent infections established (Sawtell 1998), equivalent latency was present in these groups at 42 weeks pi. Therefore, these data clearly demonstrate that the LAT locus provides one or more functions that serve to maintain the frequency of reactivation following stress in the TG over time. Furthermore, this reduced frequency of reactivation (infectious virus production) is the result of fewer neurons exiting latency (viral protein positive), which rules out the possibility that a downstream block in the replication cycle was underlying the reduced frequency of reactivation.

The above experiment revealed a previously unknown role for the LAT locus in the long-term maintenance of a stable reactivation frequency. We next sought to confirm this effect of the LAT locus using an alternate strategy to reduce the time required for the experiment. We previously showed statistically significant HS-induced reactivation from latency as early as 9 days pi, but the results were complicated by the presence of viral protein positive neurons in non-induced TG (Sawtell 2003). Several characteristics of in vivo infection with viruses lacking a functional thymidine kinase suggested that this background could be a suitable system to examine the role of the LAT locus early after infection. First, in the absence of TK function, HSV does not efficiently replicate in the TG, and there is no evidence of cell to cell spread of virus within the TG, consistent with the absence or extremely low levels of infectious virus recovered (Field and Wildy 1978; Tenser et al. 1979; Jacobson et al. 1993; Sawtell et al. 2006). Despite this, latent infections are established, and exit from latency (expression of viral proteins) can be detected following HS (Sawtell et al. 2006; Thompson et al. 2009).

LAT null plus thymidine kinase (TK), null double mutants were generated to test in the mouse model. Briefly, the TK knockout cassette we previously used to make the virus tBTK− (Pyles and Thompson 1994) was inserted into our prototypical LAT null mutant 17AH (Thompson and Sawtell 1997). In this mutation cassette, a copy of the *Escherichia coli* LacZ gene driven by the SV40 promoter is inserted at 47,563 bp in the viral genome completely eliminating TK activity while not perturbing other viral genes including UL24 (Pyles and Thompson 1994). Three independently derived dual mutants were purified to homogeneity (isolates 4–1–23, 5–2–1, and 6–9–1) as indicated by genomic structures, PCR analysis, resistance to acyclovir, and "blue plaque" assays in which ≥1,000 plaques were viewed for beta galactosidase (β-gal) activity (not shown).

Groups of mice were infected on scarified corneas with $1\times10^6$ pfu of either 17-AHTK− or 17LAT+TK− as detailed (Thompson and Sawtell 2001, 2006; Sawtell et al. 2006). All three independent isolates displayed similar replication kinetics in vivo (not shown). These viruses achieved similar titers in the eye. However, in the TG, titers of infectious virus were very low (about 3–4 orders of magnitude less than would be found in wild-type infected TG), although levels detected in mice infected with 17AHTK- (LAT null) were greater than those detected in the 17LAT+TK−-infected animals (Fig. 3a; isolate 4–1–23). The severe replication defect in TG is a phenotype common to most true TK null mutants (Horsburgh et al. 1998). Virus recovered from mouse TG at 4 days pi was resistant to acyclovir and retained β-gal activity. No virus was detected in any of the animals on days 6 and 8 pi (not shown). On day 9 pi, the remaining mice were subjected to hyperthermic stress, which induces reactivation of HSV in vivo (Sawtell and Thompson 1992a). This experiment was performed with two different 17AHTK− isolates (4–1–23 and 5–2–1) with similar results and shown are the results obtained with isolate 17AHTK− (4–1–23). Whole ganglion immunohistochemistry (WGIHC) was employed to detect neurons positive for viral proteins at 22 h post-HS as previously described (Thompson and Sawtell 1997; Sawtell 2003; Thompson et al. 2009). In the untreated group, TG neurons expressing viral proteins were

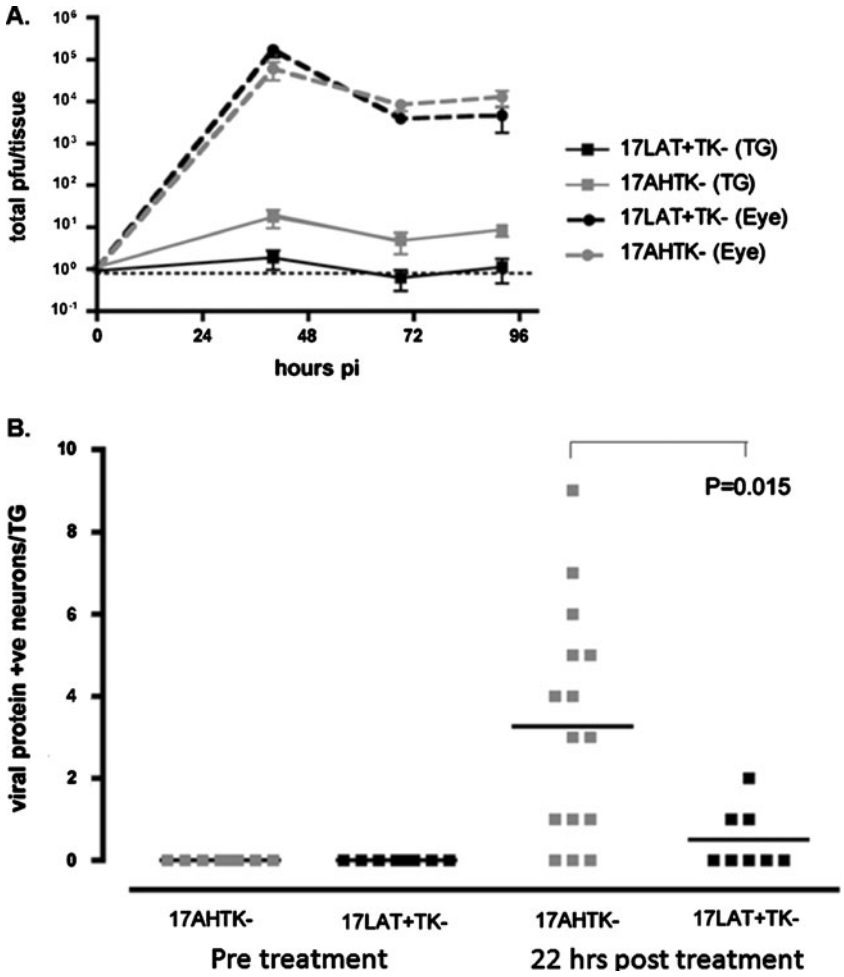


**Fig. 3** In mice infected with a LAT deletion mutant the permisivity of trigeminal neurons is increased. **a** Mice were infected via scarified corneas with $1\times10^6$ pfu of 17LAT+TK− or 17AHTK− (LAT null and TK null). **b** Groups of mice were maintained for 9 days pi and one half of the mice were subjected to hyperthermic stress. In the absence of stress, no virus was detected in any of the TG. Following HS, 12/15 TG infected with the LAT null TK null double mutant contained positive neurons, whereas 3/8 TG infected with the LAT+TK− virus contained positive neurons. The difference in the means of these groups was significant ($p$= 0.015 unpaired student $t$ test)

not detected (0/7 and 0/10 17AHTK− and 17LAT+TK− respectively; Fig. 3b). Following HS, 3/8 TG infected with the LAT+TK− mutant contained a single neuron in each of two TG and two neurons in a single TG positive for viral proteins. In contrast, 12/15 TG were positive (range=1–9 positive neurons) in the LAT null TK null-infected mice, and this difference was significant ($p$=0.015 unpaired $t$ test; Fig. 3b). Thus, using this experimental system, it is possible to detect a repressive effect conferred by the LAT locus on the activation of the lytic cycle in neurons as early as day 9 pi. These findings are consistent with the function of this locus in enhancing the number of latent infections (Sawtell and Thompson 1992a, b; Thompson and Sawtell 1997, 2001; Perng et al. 2000). However, whether this represents a single or multiple mechanisms is not known.

How the LAT locus accomplishes this task is also not yet known. It has been known for nearly two decades that the LAT locus exerts a negative influence on lytic viral gene expression (Sawtell and Thompson 1992a; Thompson and Sawtell 2001; Garber et al. 1997; Thompson and Sawtell 1997, 2001). Some groups suggest that viral proteins are expressed during latency, but their functions are not known (Green et al. 1981; Doerig et al. 1991; Thomas et al. 1999, 2002; Perng et al. 2002; Henderson et al. 2009; Jaber et al. 2009). It has been suggested that the stable 2 and 1.5 kb LAT introns might serve as a natural anti-sense RNA mechanism to inhibit the production the immediate early (IE) transactivation protein ICP0 (Stevens et al. 1987), this has been demonstrated in transient assays (Farrell et al. 1991) and may account for our observation of a post-transcriptional constraint on ICP0 production mediated by the LAT locus during reactivation from latency (Thompson et al. 2003). It is also possible that miR-H2 inhibits ICP0 expression (Umbach et al. 2008), although that remains to be demonstrated. Silencing RNAs based on the sequence of miR-H6 have been shown to inhibit the production of the IE transactivator protein ICP4 in transfection studies (Umbach et al. 2008), but we have not been able to detect silencing activity of miR-H6 expressed in an inducible cell line on luciferase gene targets containing the putative miR-H6 binding site (data not shown). Still, this microRNA, or others encoded in the LAT locus and expressed during latency, may regulate viral and/or host protein expression in as yet unknown ways to modulate reactivation frequency.

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**Conflict of interest** All authors declare that there are no conflicts of interest.

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