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Increased neurovirulence and reactivation of the herpes simplex virus type 1 latency associated transcript (LAT) negative mutant dLAT2903 with a disrupted LAT miR-H2

Xianzhi Jiang[1], **Don Brown**[1], **Nelson Osorio**[1], **Chinhui Hsiang**[1], **Lbachir BenMohamed**[1,2,3,5], and **Steven L. Wechsler**[1,5,6]

[1]Virology Research, Gavin Herbert Eye Institute and Department of Ophthalmology, University of California Irvine, School of Medicine, Irvine, California 92697

[2]Laboratory of Cellular and Molecular Immunology, Gavin Herbert Eye Institute and Department of Ophthalmology, University of California Irvine, School of Medicine, Irvine, California 92697

[3]Chao Family Comprehensive Cancer Center, University of California Irvine Medical Center, Irvine, California 92868-32013

[4]Institute for Immunology, University of California Irvine, School of Medicine, Irvine, California 92697

[5]Department of Microbiology and Molecular Genetics, University of California Irvine, School of Medicine, Irvine, California 92697

[6]Center for Virus Research, University of California Irvine, Irvine, California 92697

Abstract

At least six microRNAs (miRNAs) appear to be encoded by the latency associated transcript (LAT) of herpes simplex virus type 1 (HSV-1). The gene for ICP0, an important immediate early (IE) viral protein, is antisense to, and overlaps with, the region of LAT from which miRNA H2 (miR-H2) is derived. We recently reported that a mutant (McK-ΔH2) disrupted for miR-H2 on the wild type HSV-1 strain McKrae genomic background has increased ICP0 expression, increased neurovirulence, and slightly more rapid reactivation. We report here that HSV-1 mutants deleted for the LAT promoter nonetheless make significant amounts of miR-H2 during lytic tissue culture infection, presumably *via* readthrough transcription from an upstream promoter. To determine if miR-H2 might also play a role in the HSV-1 latency-reactivation cycle of a LAT negative mutant, we constructed dLAT-ΔH2, in which miR-H2 is disrupted in dLAT2903 without altering the predicted amino acid sequence of the overlapping ICP0 open reading frame. Similar to McK-ΔH2, dLAT-ΔH2 expressed more ICP0, was more neurovirulent, and had increased reactivation in the mouse TG explant induced reactivation model of HSV-1 compared to its parental virus. Interestingly, although the increased reactivation of McK-ΔH2 compared to its parental wt virus was subtle and only detected at very early times after explant TG induced reactivation, the

Corresponding author: Steven L Wechsler, University of California Irvine, Ophthalmology Research, 843 Health Sciences Road, Hewitt Hall (Building 843), Room 2012, Irvine, California 92697. Wechsler@uci.edu.

CONFLICT OF INTEREST

All of the authors declare that they have no conflict of interest.

increased reactivation of dLAT-ΔH2 compared to its dLAT2903 parental virus appeared more robust and was significantly increased even at late times after induction. These results confirm that miR-H2 plays a role in modulating the HSV-1 reactivation phenotype.

INTRODUCTION

Herpes Simplex Virus type 1 (HSV-1) infection is widespread in human populations and causes a wide range of diseases throughout life. In the U.S., herpes simplex encephalitis (HSE) is the leading cause of sporadic lethal encephalitis in immune competent individuals, with an untreated death rate of ~70%[1,2]. Even with antiviral therapies and greatly improved noninvasive diagnostic procedures, mortality remains unacceptably high (~20%). Additionally, over 50% of survivors have significant neurological deficits. Herpes simplex stromal keratitis (HSK), an immunopathological disease, is the most frequent serious viral eye infection in developed countries and is the leading cause of corneal blindness due to an infectious agent[3,4]. In addition, there is an increasing proportion of genital herpes cases associated with HSV-1. Most HSE, HSK and genital herpes are due to reactivation of HSV from latency, rather than to primary acute infection. Following ocular or oro-facial primary infection, HSV-1 ascends through axons and establishes latency throughout life in sensory neurons of the trigeminal ganglia (TG). To date, there is no licensed therapeutic vaccine that can effectively stop or reduce HSV-1 reactivation from latently infected sensory ganglia. Current long term anti-viral drug therapies (e.g. Acyclovir and derivatives) reduce recurrent ocular disease by only ~40%, and do not eliminate virus reactivation [5].

During HSV-1 latency, the HSV-1 Latency-Associated Transcript (LAT), is the only viral gene that is consistently detected as being abundantly transcribed [6,7]. The primary LAT transcript is 8.3 kb [8,9]. It gives rise to a family of LAT RNAs (LATs) including a very stable 2 kb LAT that appears to be an intron spliced from the primary transcript [10]. LAT plays an important role in the HSV-1 latency/reactivation cycle, since compared to LAT$^{(-)}$ mutants, LAT$^{(+)}$ viruses (i.e., wild type HSV-1) have a significantly higher spontaneous reactivation phenotype *in vivo* in rabbits and significantly higher *ex vivo* induced reactivation in the mouse TG explant reactivation model [11–14]. Theoretically, LAT could increase the HSV-1 reactivation phenotype by enhancing establishment [15–18] and/or maintenance of latency [19], be involved in the reactivation stage [20], or both. Since LAT overlaps the important immediate early ICP0 gene, it has long been speculated that suppression of the ICP0 gene may play a role in how LAT enhances the HSV-1 reactivation phenotype [21,22]. Downregulation of ICP0 by LAT may contribute to both establishment and maintenance of latency, while downregulation of LAT may trigger reactivation by derepression of ICP0 expression.

LAT's anti-apoptosis activity also appears to play a significant role in the reactivation phenotype [23–30]. The wt (i.e., LAT$^{(+)}$-like) high reactivation phenotype can be restored to a LAT$^{(-)}$ mutant by substitution of various anti-apoptosis genes in place of LAT[25,27–29]. LAT also has immune evasion properties including decreasing and/or delaying interferon production, promoting exhaustion of TG-resident HSV-specific CD8^{+} T cells, blocking granzyme B killing by CD8^{+} cytotoxic T cells, increasing herpesvirus entry mediator

(HVEM) expression, and inhibiting phenotypic and functional maturation of dendritic cells[31–36]. These may also contribute to how LAT enhances HSV-1 latency and reactivation from sensory ganglia. However, the underlying molecular mechanisms remain to be fully elucidated.

Recently, 8 “LAT” microRNAs (miRs H1 to H8) mapping in or near the LAT locus, were reported[37,38]. Six of these overlap the primary LAT transcript (miRs H2, H3, H4, H5, H7 and H8) and are assumed to be derived from LAT. Two additional miRNAs (miRs H1 and H6) are located upstream of the LAT start site. miR-H2 maps within the LAT transcript, is expressed in the LAT direction, and overlaps part of the major exon of the HSV-1 ICP0 gene in an antisense orientation. ICP0, an immediate early (IE) gene, is critical for transactivation of HSV-1 early and late genes. Downregulation of ICP0 by a LAT antisense mechanism has long been envisioned as a mechanism by which LAT might regulate latency/reactivation[6,7], but evidence for such antisense downregulation of ICP0 has not been reported. Interestingly, miR-H2 was recently reported to downregulate ICP0 translation, but not transcription, in a transient transfection assay[38].

Recently, by using codon redundancy, we were able to disrupt and KO the miR-H2 nucleotide (nt) sequence without altering the predicted amino acid sequence of the overlapping ICP0 open reading frame (ORF)[39]. This miR-H2 mutant, designated McK-ΔH2, was made on a wild type (wt) HSV-1 strain McKrae background. Compared to its wt McKrae parental virus, this mutant: (*i*) overexpresses ICP0; (*ii*) has increased neurovirulence based on survival of Swiss Webster mice following ocular infection; and (*iii*) reactivates slightly faster from latency in the mouse *ex vivo* explant induced reactivation model.

The present study extends those finding by showing that: (*i*) the six LAT miRNAs that appear to be derived from the primary LAT transcript continue to be made in tissue culture, even in the absence of the LAT promoter. This is likely the result of readthrough transcription from an upstream promoter [40]. Based on this new finding it was of interest to determine if disruption of miR-H2 on a LAT negative background (*d*LAT2903 which is deleted for the LAT promoter and the first 1,677 nucleotides of the primary transcript) would have properties similar to McK-ΔH2; (*ii*) similar to McK-ΔH2, compared to its parental virus, *d*LAT-ΔH2 overexpressed miR-H2 in tissue culture and had increased neurovirulence and reactivation in Swiss Webster mice; (ii) compared to their respective parental viruses, the increased reactivation of dLAT-ΔH2 was much more robust than that of McK-ΔH2. Altogether, the findings in this report extend and confirm our recent findings that miR-H2 functions to decrease HSV-1 pathogenesis and to help maintain HSV-1 latency.

MATERIALS AND METHODS

Cell lines

Rabbit skin (RS) cells were maintained in Eagle minimal essential medium (MEM) with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 10% fetal bovine serum (Promega Scientific), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, St. Louis, MO).

Viruses

dLAT-ΔH2 and its dLAT2903 parental virus were triply plaque purified and passaged only one or two times in rabbit skin (RS) cells prior to use. dLAT2903 is a LAT null mutant that is deleted from LAT nts −161 to +1667 and is thus missing the core LAT promoter and the first 1,667 nts of the primary transcript. dLAT2903 was derived from wild type (wt) HSV-1 strain McKrae[12].

Construction of dLAT-ΔH2

dLAT-ΔH2 was constructed by homologous recombination following co-transfection of infectious purified HSV-1 dLAT2903 genomic DNA with a plasmid containing the 21 nt alterations of interest along with sufficient flanking HSV-1 DNA sequences for efficient recombination using our standard procedures[12,25,27,29,41–47] and as previously described for construction of McK-ΔH2[39]. Briefly, 21 of the 75 H2 miRNA precursor nts were changed in a way that does not change the predicted amino acid sequence of the overlap ICP0 ORF. A XhoI restrict enzyme site was also introduced into the altered H2 miRNA precursor sequence (Fig. 3). A 469 nt sequence corresponding to the LAT sequence containing the modified H2 miRNA precursor was synthesized commercially and cloned into pUC57 between appropriate flanking sequences (Genescrip Corp.). This synthesized LAT DNA was further cloned into plasmid pBSK-LAT4.7. This contains a NotI restriction fragment insert of 4,678 nts from the LAT/miR-H2 region of dLAT2903 corresponding to nt 118442 (LAT nt (−)359) to nt 124948 (LAT nt +6,147) of the wt McKrae genome, but with the same 1,828 nt deletion as dLAT2903. This generated the ΔH2 targeting construct, pdLATH2mut, containing the altered miR-H2 precursor region flanked by approximately 1600 and 3000 nts of the dLAT2903 genomic sequence. pdLATH2mut was co-transfected with infectious purified HSV-1 dLAT2903 genomic DNA into RS cells. The virus dLAT-ΔH2 was generated by homologous recombination. Viruses from the co-transfection were plated, and isolated plaques were picked and screened for the altered H2 sequence by restriction digestion and Southern analysis. Selected plaques were plaque purified for 6 rounds and reanalyzed by restriction digestion and Southern analysis to ensure that both copies of LAT contained the altered H2 sequence. A final plaque was purified and designated dLAT-ΔH2.

Mice

Eight- to 10-week-old Swiss-Webster female mice (Jackson Labs) were used. Viral infections were done without corneal scarification as we previously described[29,44,45]. All animal work was conducted according to relevant national and international guildelines and was approved by the University of California Irvine IACUC (approval number 2002–2400). Humane endpoints were used during the survival studies. Following ocular infection mice are monitored daily for 14 days. Animals surviving to this time are no longer susceptible to HSV-1 induced encephalitis and death. If a mouse exhibits neurological symptoms (usually partial paralysis of a hind limb) or any behavior such as mild seizures that from experience we know will result in death if left untreated, the mouse is euthanized immediately and counted as having been lost to the HSV-1 infection. Our experience is that we are able to euthanize approximately 95% of the mice lost to infection. The other 5% die suddenly

without prior symptoms. Mice were euthanized by CO_2 inhalation followed by cervical dislocation to ensure that they are dead. No analgesics or anesthetics were used. Anesthetics were not needed because no surgical of painful procedures were performed (mice are infected with virus as eye drops without prior corneal scarification). Analgesics were not used because they can interfere with the outcome of the infection.

Titration of virus in tears of infected mice

Tear films were collected from both eyes of 10 mice per group on days 3, 5, and 7 post infection (p.i.) using a Dacron-tipped swab. Each swab was placed in 0.5 ml of tissue culture medium and squeezed, and the amount of virus was determined by a standard plaque assay on RS cells.

TG explant induced reactivation assay

Mice were sacrificed at 30 days p.i., and individual trigeminal ganglia (TG) were removed and cultured in tissue culture medium as we described previously[45]. Aliquots of medium were removed from each culture daily for up to 14 days and plated on indicator cells (RS cells) to assay for the appearance of reactivated virus. As the medium from the explanted TG cultures was plated daily, the time at which reactivated virus first appeared in the explanted TG cultures could be determined.

DNA extraction and PCR analysis for HSV-1 gB DNA

DNA was isolated from homogenized individual TG using a DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504) according to the manufacturer's instructions. PCR analysis was done using gB specific primers (forward, 5′-AACGCGACGCACATCAAG-3′; reverse, 5′-CTGGTACGCGATCAGAAAGC-3′; and probe, 5′-FAM-CAGCCGCAGTACTACC-3′, where FAM is 6-carboxyfluorescein). All the primers and probe were synthesized by Sigma-Aldrich Corp. The amplicon length for this primer set is 72 bp. Relative copy numbers for the gB DNA were calculated using standard curves.

Stem-loop RT-PCR analysis for LAT miRNAs

The expression of LAT miRNAs were assayed by quantitative stem-loop RT-PCR as previously described[38] using similar primers synthesized by Sigma. Total RNA was extracted using a Direct-zol™ RNA MiniPrep Kit (Zymo Research). RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) and miRNA-specific RT primers. Aliquots of cDNA were assayed on an ABI 7900HT fast real-time PCR system (Applied Biosystems).

Western blots

Total cell extracts were separated by Novex 4–20% tris glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Life Technologies) and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with mouse monoclonal anti-ICP0 (EastCoast Bio.) at a 1:8,000 dilution, or polyclonal rabbit anti-GAPDH antibody at a 1:10,000 dilution (GeneTex, Irvine, CA) and then washed, and the antibody bound to the blots was visualized by chemiluminescence (Thermo Scientific) with goat anti-mouse

immunoglobulin G (IgG) or goat anti-rabbit IgG conjugated to horseradish peroxidase according to the instructions of the manufacturer (Thermo Scientific).

Southern blots

Briefly, viral DNA was double-digested with BamHI and XhoI. The restriction fragments were separated in a 0.8% agarose gel, transferred to Nylon membrane, rinsed in 2×SSC (1×SSC is 0.15M NaCl plus 0.015M sodium citrate) for 5 min, and cross-linked to the membrane by UV light. DNA-DNA hybridization was performed with ^{32}P-labeled probes.

RESULTS

Successful detection of six HSV-1 LAT miRNAs in tissue culture cells

To ensure that we could detect the same six miRNAs that were originally reported to overlap the primary 8.3 kb LAT transcript[38] we reproduced their findings[37], using the same virus (HSV-1 strain KOS) and cells (SY5Y cells). Monolayers of SY5Y cells were infected with KOS at an MOI of 10 pfu/cell. At 18 h p.i. RNA was purified and stem-loop quantitative RT-PCR was done as described in *Materials & Methods*. The results are presented using the convention of fold over background, as previously reported[37] (Fig. 1). We found the relative amounts of each LAT miRNA to be similar to those previously reported[37], suggesting that we detected the same miRNAs.

SY5Y and CV-1 cells infected with LAT deletion mutants express all six LAT miRNAs in the absence of the LAT promoter

To confirm that our standard wt HSV-1 strain McKrae also made the same LAT miRNAs, we infected SY5Y cells with McKrae as in Fig. 1 (Fig. 2A). As a negative control, SY5Y cells were similarly infected with dLAT2903, a LAT mutant deleted for the core LAT promoter and the first 1,667 nts of LAT, that makes no detectable large LAT transcripts[12] and was expected to be negative for the LAT miRNAs. As expected, all 6 LAT miRNAs were detected in SY5Y cells infected with McKrae (Fig. 2A; black bars) and the relative amounts were similar to that seen with KOS (compare to Fig. 1). Surprisingly, the miRNAs were also seen in SY5Y cells infected with dLAT2903 (Fig. 2A; open bars) and the amounts appeared to be similar to, or in some cases, slightly lower than with McKrae. We then examined a different LAT promoter deletion mutant 17ΔPst[48] constructed from HSV-1 strain 17syn+ using a different cell line (CV-1 cells). Again, the overall pattern of LAT miRNA expression was similar with the LAT deletion mutant and its wt parental virus Fig. 2B). 17ΔPst has a relatively small deletion of the primary LAT promoter region from LAT nt (−)137 to LAT nt +65 [48], while dLAT2903 has a large deletion encompassing LAT nucleotides (nts) (−)161 to +1667[12]. dLAT2903 is deleted for both the primary LAT promoter and a secondary putative LAT promoter (LAP2)[49] located approximately 630 nts downstream of the start of the primary LAT transcript. These results indicate that in tissue culture the LAT miRNAs can be made in the absence of any known LAT promoter. This could be the result of either readthrough transcription from an upstream promoter or transcription from one or more previously unrecognized internal LAT promoters. Addressing this question is outside the scope of this report.

LAT miR-H2 is approximately 25% less abundant in tissue culture cells infected with LAT promoter deletion mutants

Fig. 2C shows the miR-H2 results from Fig. 2A plotted on a linear rather than a semi-log graph. This visually enhances the difference between the McKrae and dLAT2903 results. Expression of miR-H2 by dLAT2903, although still high relative to McKrae (~6,000 compared to ~8,000 fold over background), was significantly reduced (P=0.002). Expression of miR-H2 by 17ΔPst was also reduced by approximately 25% compared to its wt 17syn+ parent (P=0.038). Thus, although statistically less miR-H2 was made by LAT promoter deletion mutants, significant amounts of miR-H2 were still present.

To determine the effect of a LAT promoter deletion mutant on expression of miR-H2 during latency, C57BL/6 mice were infected with $2x10^5$ pfu/eye of wt McKrae or dLAT2903. Infected trigeminal ganglia (TG) were harvested 30 days post infection when latency was well established. Total RNA was isolated and the relative amount of miR-H2 was determined as above. miR-H2 was detected in most of the TG from mice latently infected with McKrae, but was not detected in any of the TG from mice latently infected with dLAT2903 (data not shown).

Thus, very little or no LAT miR-H2 (and presumably other LAT miRNAs) was made by this LAT promoter deletion mutant during latency. This supports the hypothesis that expression of the LAT miRNAs by dLAT2903 and 17ΔPst in tissue culture was due to readthrough transcription from an upstream promoter and that this promoter is turned off (along with all the other HSV-1 genes except LAT) during neuronal latency. We recently showed that McK-ΔH2, a wt McKrae based mutant disrupted for miR-H2 had increased neurovirulence and increased reactivation in the mouse explant TG model of induced reactivation [(39)]. Since miR-H2 continued to be expressed in the absence of the LAT promoter in tissue culture, we decided to examine the effect of disrupting miR-H2 on a dLAT2903 genomic background.

Codon redundancy allows disruption of miR-H2 without altering the ICP0 amino acid sequence

As we previously described for construction of McK-ΔH2, miR-H2 overlaps the HSV-1 ICP0 open reading frame (ORF) in an anti-sense direction (Fig. 3A). Thus, deleting the LAT region encoding miR-H2 would also delete and disrupt the ICP0 ORF. Since ICP0 is an important IE gene that is a major factor in efficient virus replication, this would complicate analysis of such a deletion mutant. Thus, it was important to disrupt miR-H2 without altering the predicted amino acid sequence of ICP0. To overcome this obstacle, we made use of codon redundancy, as we did for construction of McK-ΔH2, to significantly alter the nt sequence of miR-H2 without altering the predicted amino acid sequence of the overlapping region of the ICP0 gene. Fig. 3 shows: (A) the location of miR-H2 relative to LAT and ICP0; (B) the relative location of the deletion in dLAT2903; (C) the approach use to construct the dLAT-ΔH2 mutant; and (D) the wt and altered (ΔH2) nt sequences and the predicted ICP0 amino acid sequence corresponding to the altered region, which is identical for the wt and ΔH2 sequences. In the altered nt sequence 21 of the 75 miR-H2 precursor nts (28%) were changed. These changes do not alter the predicted amino acid sequence of the corresponding region of ICP0. The sequence was synthesized commercially. We previously

showed [39] that: 1) these nt changes dramatically alter the predicted RNA structure; 2) the altered sequence no longer produces a miRNA; 3) if a miRNA were made it would not function correctly; and 4) the altered miR-H2 sequence is no longer able to downregulate ICP0 protein expression in a co-transfection assay. The miR-H2 disrupted mutant on a dLAT2903 background was constructed using procedures we previously described for construction of other HSV-1 mutant viruses[12,29,43] and specifically identically to that recently used to make McK-ΔH2 (a miR-H2 disrupted mutant on a wt McKrae background) [39], as described in *Materials & Methods*. Southern blot analysis similar to our previous Southern blot analysis of McK-ΔmiR-H2 [39], confirmed that the dLAT-ΔH2 genomic structure was as expected (Fig. 3E). The resulting mutant, dLAT-ΔH2, is therefore identical to McK-ΔH2 except that the LAT promoter and the first 1.6 kb of LAT are deleted.

Increased expression of ICP0 in tissue culture cells infected with the dLAT-ΔH2 mutant

We previously showed that RS cells infected with McK-ΔH2 had increased expression of ICP0 compared to wt McKrae or its marker rescued virus McK-ΔH2Res [39]. To determine if dLAT-ΔH2 also expressed elevated levels of ICP0, RS cells were infected at an MOI of 5 and the monolayers were collected 2, 4, 6, and 10 hrs post infection (p.i.). Western blots were run using cell extracts as detailed in *Materials & Methods* and as we previously described [39]. Blots were analyzed using mAb specific for ICP0 (Fig. 4). GAPDH was included as a loading control. As expected, more ICP0 was present at early times p.i. with dLAT-ΔH2 compared to parental dLAT2903. This is most apparent at the 4 and 6 hrs p.i. time points. This profile is very similar to what was previously seen with McK-ΔH2 compared to its parental virus [39]. Thus, like McK-ΔH2, dLAT-ΔH2 appeared to express more ICP0 protein than its parental virus. Since we previously showed that the nt alterations used to disrupt miR-H2 do not increase stability of the altered ICP0 mRNA or the translation efficiency of plasmids [39], these results strongly suggest that miR-H2 normally downregulates expression of ICP0.

Normal replication of the dLAT-ΔH2 mutant in tissue culture in vitro and in mouse eyes in vivo

To ensure that any phenotype exhibited by dLAT-ΔH2 was not due to abrogation of ICP0 function, we examined replication of dLAT-ΔH2. Although ICP0 is not an essential virus protein, ICP0 is an important IE gene and ICP0 mutants replicate poorly when cells are infected at a low MOI[50]. Therefore, we infected RS cells with dLAT-ΔH2 vs dLAT2903 using an MOI of 0.01 (Fig. 5A). Replication of dLAT-ΔH2 was at least as efficient as dLAT2903. This confirmed that ICP0 in dLAT-ΔH2 was fully functional, since replication of dLAT-ΔH2 was expected to be defective if ICP0 function was abnormal.

We then examined dLAT-ΔH2 replication during acute infection of mouse eyes. Swiss Webster mice were infected with our normal dose of $2x10^5$ pfu/eye and tears were collected at the times indicated in Fig. 5B, by swabbing eyes as described in *Materials & Methods*. The amount of virus was determined by standard plaque assays on RS cells. Replication of dLAT-ΔH2 and its parental dLAT2903 virus were similar. Thus, dLAT-ΔH2 also appeared to replicate normally in mouse eyes.

The dLAT-ΔH2 mutant has increased neurovirulence compared its parental dLAT2903 virus

Following ocular infection of Swiss Webster mice with $2x10^5$ pfu/eye using eye drops without corneal scarification some mice succumb to viral encephalitis as we previously described[14]. Most mice that succumb to infection die suddenly from encephalitis, while some develop significant neurological symptoms that based on previous experience would shortly lead to death. These mice are euthanized and are considered to have succumbed to the infection. We define neurovirulence here as the ability of the virus to kill mice by encephalitis following ocular infection, without distinguishing between neuroinvasion (the ability of the virus to get to the CNS) and the formal definition of neurovirulence (the ability of the virus to kill mice after it gets to the CNS). Mice were infected with dLAT-ΔH2 or dLAT2903 and survival was monitored for approximately 2 weeks. The results were analyzed using a Kaplan-Meier survival curve analysis (Fig. 6). dLAT-ΔH2 appeared more neurovirulent than its parental dLAT2903 virus (P=0.047). This increased neurovirulence was similar to the increased neurovirulence we recently reported for McK-ΔH2, and confirms that disrupting miR-H2 results in increased neurovirulence in Swiss Webster mice, regardless of the presence or absence of the LAT promoter.

The dLAT-ΔH2 mutant has an increased reactivation phenotype compared its parental dLAT2903 virus

In separate experiments, Swiss Webster mice were ocularly infected with either $2x10^4$ or $2x10^5$ pfu/eye of dLAT-ΔH2 or dLAT2903. Thirty days p.i., when latency was well established, surviving mice were euthanized and individual trigeminal ganglia (TG) were explanted into tissue culture media. The time to first appearance of reactivated virus was determined by plating an aliquot of media that was removed daily from the explanted TG media and plated on RS indicator cells. The results for the 10–11 days of sampling were plotted and analyzed by Kaplan-Meier survival curve analysis. At the lower dose infection, dLAT-ΔH2 had more reactivation by this analysis than did dLAT2903 (Fig. 7A; P=0.013). Increased reactivation of dLAT-ΔH2 compared to dLAT2903 was also seen at the higher dose infection by this survival curve analysis (Fig. 7B; P=0.0003). dLAT-ΔH2 also had significantly higher reactivation at both infectious doses when analyzed by the Fisher's exact test (Fig. 6B, $2x10^4$ pfu/eye, P=0.036 and Fig. 6D, $2x10^5$ pfu/eye, P=0.018). The smaller "P" values for the $2x10^5$ dose compared to the $2x10^4$ dose are likely due to the increased power of using more mice in the higher dose experiment. The results of these experiments strongly suggest that dLAT-ΔH2 reactivated more efficiently from latency than its dLAT2903 parent virus. This is consistent with, but more robust (see Discussion), than our previous report for McK-ΔH2, which appeared to have increased reactivation only at very early times after TG explantation.

PCR analysis of DNA from individual TG does not detect significant differences in establishment of latency of dLAT-ΔH2 versus dLAT2903

To determine if the increased reactivation of dLAT-ΔH2 correlates with increased latency viral load in TG, the amount of latency was examined in individual TG from mice infected as above, on day 30 pi as we previously described[42] and *Materials & Methods*. No

significant differences were detected (Fig. 8), suggesting that disruption of miR-H2 did not have a major impact on the total amount of latency established in whole TG extracts. Similar results were seen previously with McK-ΔmiR-H2[41].

DISCUSSION

In the present report we showed that disruption of LAT miR-H2 in dLAT2903 (a mutant deleted for the LAT promoter and the first 1,667 nts of LAT) increased virus reactivation in the mouse TG explant induced reactivation model. The more proper terminology is that this mutant had an increased "reactivation phenotype", because this is what we measured, regardless of whether the mechanism is directly involved in the reactivation process. We recently reported that a mutant disrupted for miR-H2 that was constructed on a wt McKrae background (McK-ΔH2) had an increased reactivation phenotype in the mouse explant TG induced reactivation model at early time points after TG explantation[41]. We described the increased reactivation phenotype of McK-ΔH2 compared to its wt parent and its marker rescued virus as "subtle". In one experiment the increased McK-ΔH2 reactivation was only seen on day 3 after TG explant, while in another experiment it was seen on days 3 and 4, but not at later times. Typically, statistical analysis of TG explant reactivation is done either: 1) by Fisher's exact test analysis of the total number of TG that produced reactivated virus vs. the total number of TG that did not produce reactivated virus by the end of the study period (usually 10 or more days after explant) as is shown in Fig. 7C and 7D above for dLAT-ΔH2; or 2) by a Kaplan-Meier survival curve analysis covering the entire study period, as shown above in Fig. 7A and 7B). When the results of our previous studies with McK-ΔH2 were analyzed by these methods no significant increase in the reactivation phenotype of McK-ΔH2 was found (unpublished results). Only by closely examining the results on each day (Fisher's exact) did we find that McK-ΔH2 had a slightly increased reactivation phenotype on day 3 or days 3 and 4. It would have been easy to overlook this subtle increase in the McK-ΔH2 reactivation phenotype at these early times. Thus, potential alterations in the reactivation phenotype of some miR-H2 mutants may go unnoticed.

While confirming that we could detect the previously reported miRNAs that overlap the LAT transcript, we found that mutants deleted for the LAT promoter (i.e., $LAT^{(-)}$ mutants that were expected to be negative controls for $LAT^{(+)}$ wt virus) also produced these LAT miRNAs in tissue culture. Since $LAT^{(-)}$ viruses have a reduced reactivation phenotype compared to their wt parental viruses, we hypothesized that an increased reactivation phenotype due to disruption of miR-H2 might be more readily detected on a $LAT^{(-)}$ background. In other words, we predicted that since the $LAT^{(-)}$ parental virus would have a lower background of reactivation than wt virus, increases in the reactivation phenotype due to disruption of miR-H2 might be easier to see. This led us to construct and analyze the dLAT-ΔH2 mutant described in this report. As we hypothesized, the effect of miRNA disruption in dLAT-ΔH2 appeared much easier to detect. Specifically in two different experiments using different initial infectious doses of virus, a statistically significant increased explant TG induced reactivation phenotype was seen for dLAT-ΔmiR-H2 by: 1) Fisher's exact analyses of the total results at the end of the study period; and 2) Kaplan-Meier survival curve analyses.

Other than the more apparent effect on the reactivation phenotype, the results for dLAT-ΔH2 were similar to those for McK-ΔH2. Specifically: 1) both mutants had increased expression of ICP0; 2) replication of both mutants in tissue culture and in mouse eyes were similar to their parental viruses; 3) both mutants had increased neurovirulence (killed more mice following ocular infection) compared to their parental viruses; and 4) neither mutant appeared to significantly alter the amount of latency established (i.e., they were each similar to their respective parental viruses). It should be noted, however, that this does completely not rule out the possibility that dLAT-ΔH2 establishes latency in more neurons than its parental dLAT2903 virus. PCR analysis of total TG extracts sometimes does not detect changes in the amount of latency established [12,51] on a per neuron basis. Occasional latently infected neurons may contain extremely large HSV-1 genome copy numbers compared to the majority of latently infected neurons in a TG. This can overwhelm detection of differences in the percent of neurons that are latently infected. Thus, it remains possible that more complex future analyses to determine the percent of latently infected neurons per TG might reveal differences between the miR-H2 KO mutant and its parental virus.

In our previous report on McK-ΔH2, the mutant was compared to its parental wt virus and to its wt marker rescued virus. The marker rescued virus was indistinguishable from the original wt virus. Marker rescued viruses are used to ensure that any phenotype seen in the mutant virus is actually due to the engineered mutation and not to some unexpected random mutation at a distant site. However, there are times when a marker rescued virus is not essential. For example, if the same mutant is independently made twice and both mutants have the same phenotype, a marker rescued virus is not needed[18]. This is because the chance of an unexpected mutation occurring at a distance site in both constructs which would each produce the same non-parental virus phenotype is very remote. In this report a marker rescued virus was not needed to confirm that the phenotypes were due to disruption of miR-miR-H2 (i.e., the engineered mutation), since: 1) we had previously shown that marker rescue of McK-ΔH2 restored the parental phenotype as expected; 2) the engineered mutation in dLAT-ΔH2 is identical to that of McK-ΔH2; and 3) McK-ΔH2 and dLAT-ΔH2 have the same phenotypes. Nonetheless, because dLAT-ΔH2 was not directly compared to it marker rescued virus in these studies it is possible although unlikely, that a lesion introduced outside the intended mutation could be responsible for one or more of the phenotypes seen.

The results in Figs. 1 and 2 were reported as “fold over background” and generated by RT-PCR in an effort to most closely reproduce the methods used in the papers that originally reported the LAT miRNAs. This allowed us to confirm that we were detecting the miRNAs that were originally reported. We used the equivalent primers and infected the same cell lines in an identical manner. Target-specific stem-loop reverse transcription primers that target mature microRNAs were used to eliminate background from precursor miRNAs or larger transcripts.

Previously we showed that a plasmid containing the altered ICP0 nt sequence and a plasmid containing the wt ICP0 nt sequence produced similar levels of ICP0 protein following transfection. This strongly suggests that transcription, translation, and mRNA stability of the altered ICP0 sequence was not significantly altered by the 21 nt replacements used to disrupt

the miR-H2 sequence. This strongly suggests that the altered ICP0 codon usage did not increase ICP0 levels independent of the miR-H2 KO in either McK-ΔH2 or dLAT-ΔH2.

Interestingly, when wt HSV-1 strain KOS was grown in Vero cells engineered to express ICP0 (cell line 0–28) no increase in virus replication or efficiency of plaque formation was seen compared to normal Vero cells[50]. This is consistent with our findings for dLAT-ΔH2 here and McK-ΔH2 previously[42] that the increased amounts of ICP0 made by these mutants did not significantly impact virus replication in tissue culture or mouse eyes. We hypothesize that the neurovirulence and reactivation assays we used in mice are more sensitive to the effect of elevated ICP0 levels than are assays for virus replication.

The results presented here, in conjunction with our previous report on McK-ΔH2, indicate that disruption of miR-H2 increases the HSV-1 reactivation phenotype. In addition to confirming our previous results with McK-ΔH2 and making it more apparent that disruption of miR-H2 results in an increased reactivation phenotype, the results reported here also strongly suggest that the mechanism by which disruption of miR-H2 virus appears to increase the reactivation phenotype acts at a time(s) other than during latency. This strongly suggests that the mechanism by which miR-H2 normally decreases the reactivation phenotype also acts at a time(s) other than during latency. In wt HSV-1, miR-H2 is made during both acute infection and during neuronal latency. Therefore phenotypic differences between McK-ΔH2 and its parental wt virus could be the result of decreased miR-H2 during the acute phase of infection, as latency is being established, during latency, during the reactivation process, and/or just after reactivation has occurred. In contrast, LAT promoter negative viruses, which in tissue culture appear to express miR-H2 by readthrough transcription from a non-latency upstream promoter, do not express miR-H2 during latency. Thus, logically, the increased reactivation phenotype resulting from disruption of miRNA in the dLAT-ΔH2 mutant cannot result from a mechanism acting during latency, since in dLAT2903 miR-H2 is not expressed at this time. Thus, the effect of disrupting miR-H2 must be due to something happening either: 1) during acute infection (perhaps more virus is made, although we were unable to detect this); 2) at the time latency is being established (i.e., before viral gene expression is completely shut off), which could increase the amount of latency established, although this was not detected; 3) once reactivation from latency is triggered, for example once the upstream promoter driving miR-H2 expression in the absence of a LAT promoter is turned back on; and/or 4) just after reactivation occurs. However, the mechanism by which disruption of miR-H2 increases reactivation (i.e., the mechanism by which miR-H2 normally decreases reactivation or helps maintain latency) cannot have its major effect during latency.

The increased reactivation phenotype of dLAT-ΔH2 strongly suggests that miR-H2 decreases reactivation and/or helps maintain latency. dLAT-ΔH2 also had increased ICP0 expression, strongly suggesting that miR-H2 normally functions to repress ICP0 expression. This is the same as our recent finding with McK-ΔH2 and is consistent with reports showing that miR-H2 can down regulate ICP0 expression in transient transfection assays with plasmids[37,38]. The lack of increased replication with dLAT-ΔH2 was similar to our findings with McK-ΔH2 and was consistent with a report of a different miR-H2 mutant which did not have altered replication in NIH 3T3 cells as judged by viral DNA levels[52].

dLAT-ΔH2 and McK-ΔH2 had increased neurovirulence in Swiss Webster mice that appeared to be a result of increase ICP0 expression. This is consistent with a recent report about mouse miRNA miR-138, which similar to miR-H2, downregulates ICP0. An HSV-1 mutant in which the target site for mouse miR-138 (on ICP0) is disrupted, has enhanced expression of ICP0 and increased encephalitis and death in mice[53]. Thus, we hypothesize that the increased neurovirulence of our dLAT-ΔH2 and McK-ΔH2 mutants is due to increased ICP0 expression in the absence of downregulation by miR-H2. Although it is tempting to conclude that the phenotypes of dLAT-ΔH2 were solely due to increased ICP0 expression, it must be kept in mind that miR-H2 may have additional, yet to be determined functions, whose loss may significantly contribute to the phenotypes seen.

Why would a mechanism evolve in HSV-1 that decreases neurovirulence and reactivation from latency? Both are likely to be beneficial for the virus and hence subject to selective pressure. Host survival is obviously increased by modulating neurovirulence. This is a common adaptation when a virus and its host co-evolve. Latency combined with sporadic reactivations can be a strong virus adaptation because latency increases host survival, virus survival, and allows the virus to “hide out” from the host immune system, while reactivations allow for spread of the virus to additional hosts. However, too much reactivation might over expose the virus to the immune system. Thus, evolutionary forces might select for viruses with decreased virulence, that can establish latency, and that have modest levels of reactivation. Thus, it is logical for miR-H2 to have evolved to modulate ICP0 thus reducing HSV-1 neurovirulence and modulating reactivation.

In summary, the findings reported here confirm and extend our previous findings that LAT miR-H2 may play a significant role in the HSV-1 latency-reactivation cycle. HSV-1 miR-H2 appears to decrease HSV-1 neurovirulence and decrease the reactivation phenotype, presumably by modulating expression of the important IE protein ICP0.

Acknowledgments

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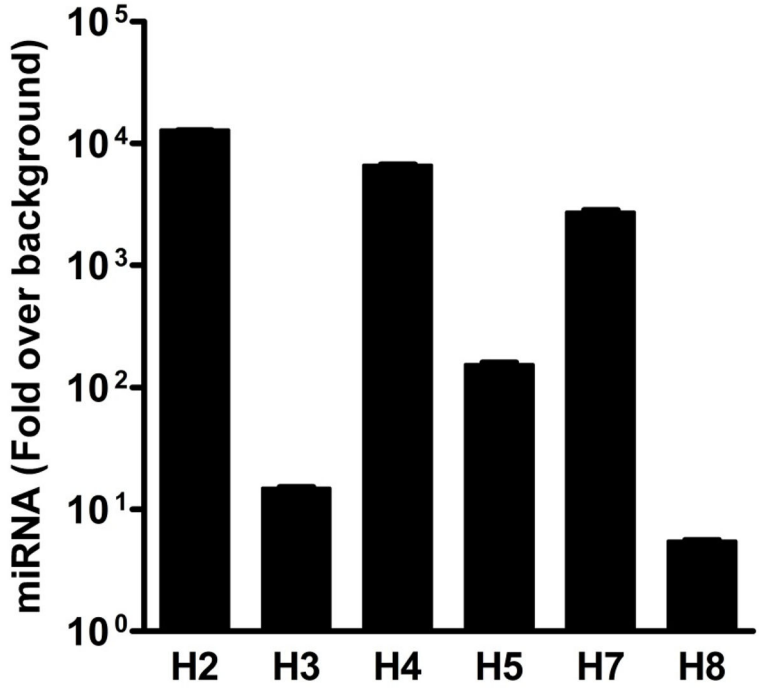

Fig. 1. Detection of six LAT encoded miRNAs

SY5Y cell monolayers were infected with KOS at an MOI of 10 pfu/cell and total RNA isolated at 18 h p.i. Stem-loop qRT-PCR was performed using primers for each of 6 LAT miRNAs that appear to be processed from the primary 8.3 b LAT transcript, as described in Materials & Methods and plotted as fold over background. The results are similar to a recent report[37]. For most of the results, the error bars are too small to see on this semi-log plot.

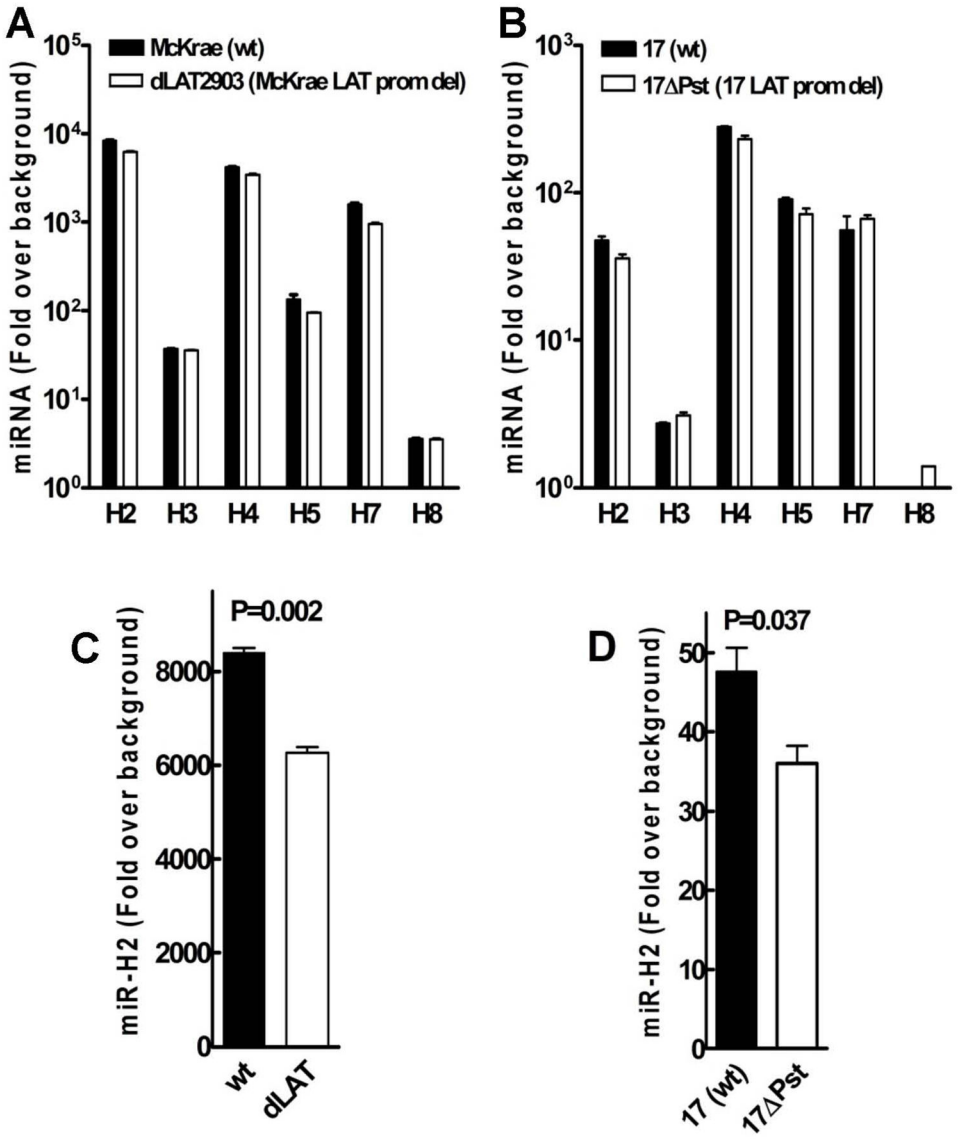

Fig. 2. LAT promoter deletion (LAT$^{(-)}$) viruses express the LAT miRNAs during acute tissue culture infection

Tissue culture cell monolayers were infected with wt McKrae, dLAT2903 (a LAT$^{(-)}$ mutant of McKrae), wt 17syn+, or 17ΔPst (a LAT$^{(-)}$ mutant of 17syn+). Infections were done at an MOI of 10 pfu/cell. Eighteen hours p.i., RNA was harvested and stem-loop qRT-PCR was performed as described in Materials & Methods. Panels: (A), SY5Y cells; (B) CV-1 cells; (C) linear plot of the miR-H2 results shown on the semi-log plot in panel A; (D) linear plot of the miR-H2 results shown in panel B allows the error bars to be seen more easily. Each bar shows the average +/− SEM of 5 to 6 replicates.

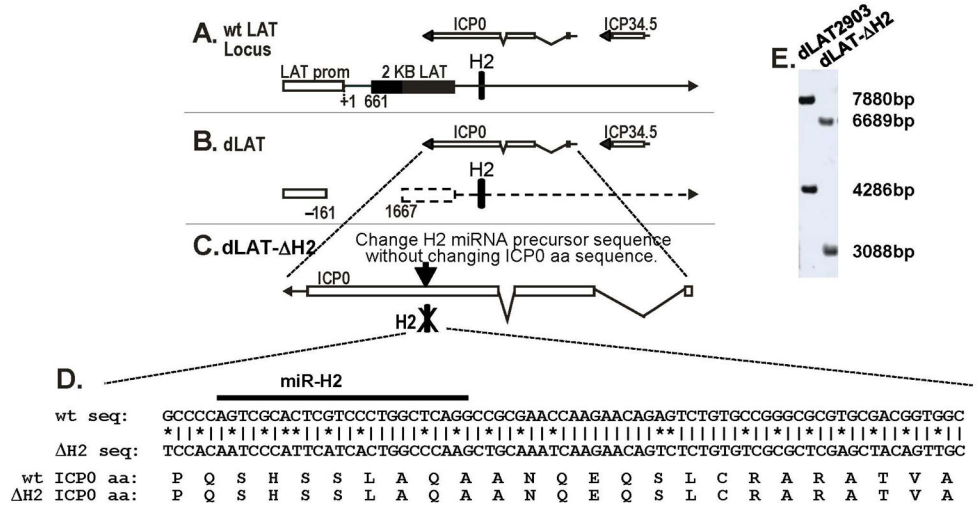

Fig. 3. Construction and genomic sequence of dLAT-ΔH2

Each of the two long repeat regions of the HSV-1 genome contains one copy of the LAT gene (not shown). For simplicity, only one LAT copy is shown. Both LAT regions are identical in each virus. Panel **(A)** Schematic representation of the LAT region in wt HSV-1. The relative locations of the LAT promoter (open rectangle), the ~8.3 kb primary LAT transcript (large arrow), the stable 2 Kb LAT intron (black rectangle), and miR-H2 (vertical solid rectangle labeled H2) are shown. The relative locations of the genes for ICP0 and ICP34.5 both of which overlap LAT in the opposite orientation, are also show. The numbers under LAT are nt positions relative to the start of LAT transcription (nt +1). miR-H2 overlaps ICP0. miR-H2 and the other LAT miRNA in this report (not show) are all located downstream of the 2 Kb LAT. **(B)** Schematic of dLAT2903, the parental LAT$^{(-)}$ virus of dLAT-ΔH2. dLAT2903 has a deletion extending from −161 to +1667 and is thus missing the core LAT promoter and the first 1,667 nts of the primary transcript. The dashed lines indicate that no large transcript corresponding to this region of LAT can be detected. **(C & D)** The region in dLAT-ΔH2 corresponding to miR-H2 and the ICP0 gene is expanded. The sequence of miR-H2 is disrupted by changing 21 of 75 miR-H2 precursor nts without changing the amino acid sequence of ICP0. **(E)** Southern blot analysis of dLAT-ΔH2. RS cells were infected and viral DNA extracted, digested with BamHI and XhoI and Southern blots performed as described in Materials & Methods. The LAT (ICP0/miR-H2) bands produced by the BamHI and XhoI digestion from the two repeat regions are of different sizes. Since altering the ICP0 sequence to KO miR-H2 introduced a XhoI restriction site at this location, both bands from dLAT-ΔH2 are smaller than the corresponding bands from dLAT2903.

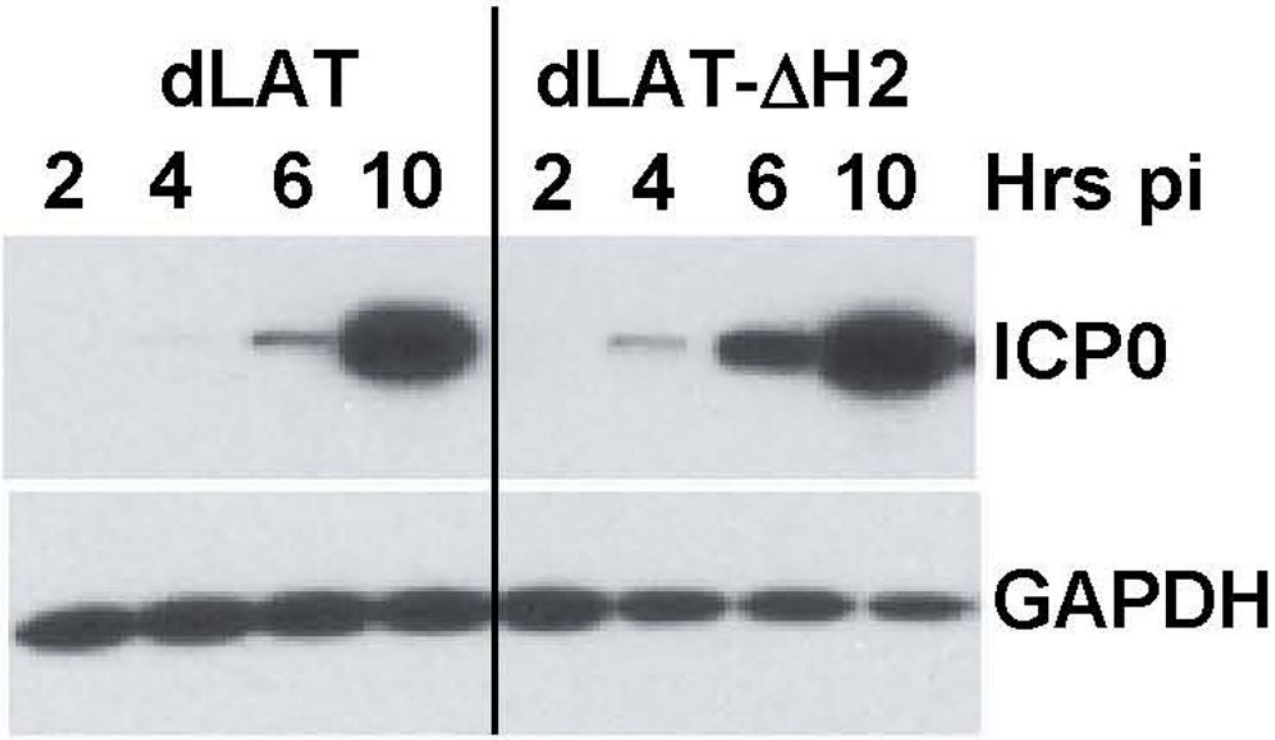

Fig. 4. Increased ICP0 protein levels expressed by dLAT-ΔH2

RS cells were infected, harvested at the times indicated post infection (Hrs pi), and Western blots were done using an anti-ICP0 mAb to detect ICP0 as described in Materials and Methods. GAPDH is a loading control.

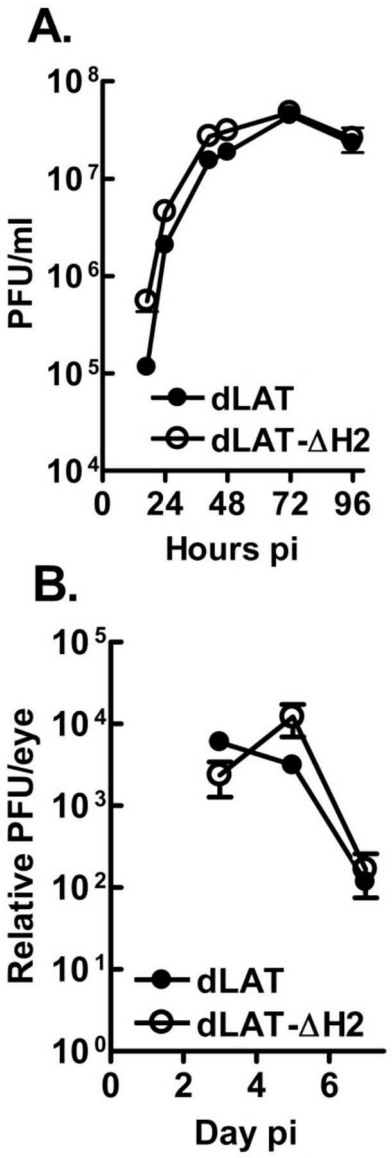

Fig. 5. Replication of dLAT-Δ H2 in tissue culture and in mouse eyes

Panel A: RS cell monolayers were infected with the indicated virus at an MOI of 0.01. At the times indicated the monolayer and tissue culture media were freeze thawed 2X and the amount of virus determined by standard plaque assays on RS cells. Each time point is the average of three replicates +/− SEM. **Panel B**: Mice were ocularly infected with 2 x 10^5 pfu/eye of the indicated virus. Tears swabs were collected on the indicated days and the amount of virus determined by plaque assays on RS cells. Each time point represents the average of 10 eyes +/− SEM.

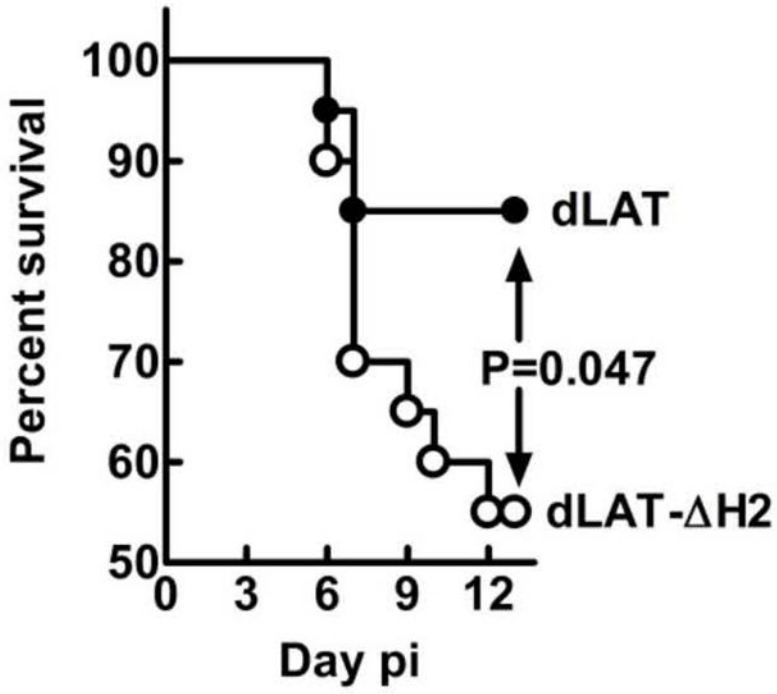

Fig. 6. Neurovirulence of dLAT-ΔH2 determined by survival of Swiss Webster mice

Mice were ocularly infected with 2 x 10^5 pfu/eye of the indicated virus. The percent of mice surviving during the first 10 days pi was determined and plotted and P value determined using a Kaplan-Meier survival curve analysis. dLAT-ΔH2: 20 mice; dLAT2903: 20 mice.

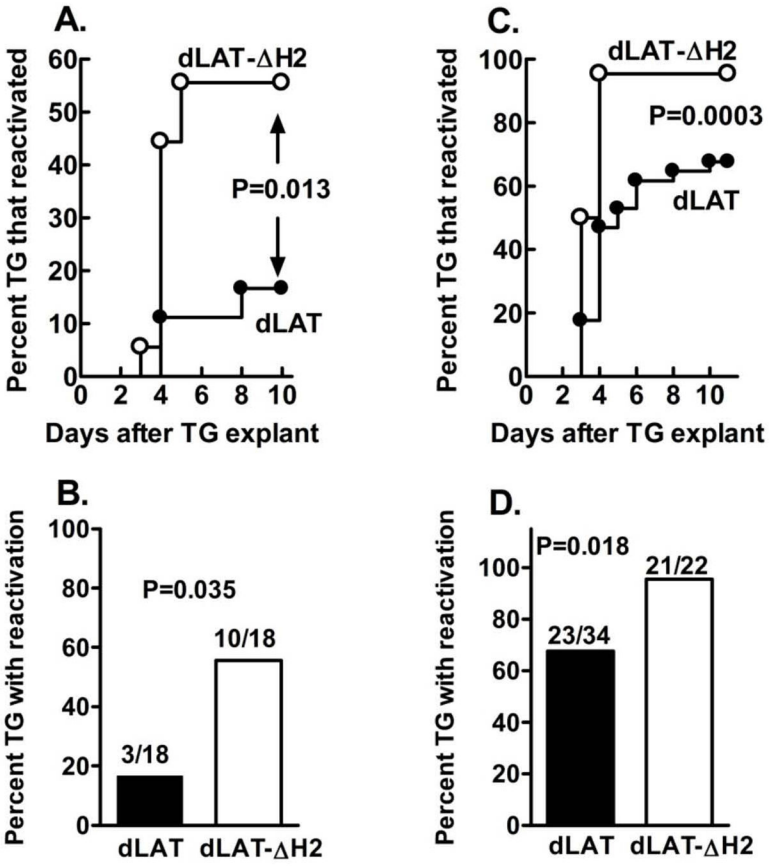

Fig. 7. Reactivation of dLAT-ΔH2

Panel A: Mice were ocularly infected with $2x10^4$ pfu/eye of the indicated virus. 30 days pi surviving mice were euthanized and TG removed for induction of reactivation by explantation into tissue culture media. Aliquots of the tissue culture media were collected daily and plated on RS indicator cells to determine the time of first appearance of reactivated virus. The cumulative percent of TG from which virus had reactivated are plotted. dLAT-ΔH2: 18 TG; dLAT2903: 18 TG. **Panel B**: Mice were infected and reactivation determined as in panel A except that eyes were infected with 10X more virus ($2x10^5$ pfu/eye). dLAT-ΔH2: 22 TG. dLAT2903:34 TG.

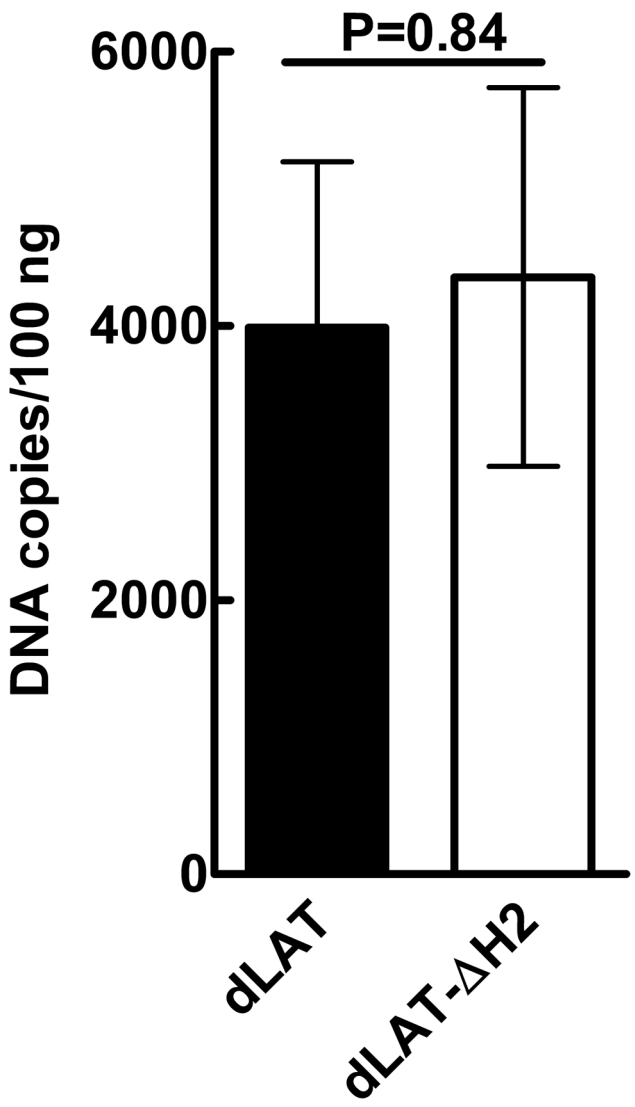

Fig. 8. Virus genome copies in latently infected TG

Mice were infected as above. 30 days pi, TG were harvested and the amount of HSV-1 genome copies in individual TG was determined by qPCR using probes specific for the HSV-1 gB gene. 6 TG per group.