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The Latency-Associated Transcript Gene of Herpes Simplex Virus Type 1 (HSV-1) Is Required for Efficient In Vivo Spontaneous Reactivation of HSV-1 from Latency

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During herpes simplex virus type 1 (HSV-1) neuronal latency, the only viral RNA detected is from the latency-associated transcript (LAT) gene. We have made a LAT deletion mutant of McKrae, an HSV-1 strain with a very high in vivo spontaneous reactivation rate. This mutant (dLAT2903) lacks the LAT promoter and the first 1.6 kb of the 5′ end of LAT. dLAT2903 was compared with its parental virus and with a rescued virus containing a restored LAT gene (dLAT2903R). Replication of the LAT mutant in tissue culture, rabbit eyes, and rabbit trigeminal ganglia was similar to that of the rescued and parental viruses. On the basis of semiquantitative PCR analysis of the amount of HSV-1 DNA in trigeminal ganglia, the LAT mutant was unimpaired in its ability to establish latency. In contrast, spontaneous reactivation of dLAT2903 in the rabbit ocular model of HSV-1 latency and reactivation was decreased to approximately 33% of normal. This decrease was highly significant ($P < 0.0001$) and demonstrates that in an HSV-1 strain with a high spontaneous reactivation rate, deletion of LAT can dramatically decrease in vivo spontaneous reactivation. We also report here that deletion of LAT appeared to eliminate rather than just reduce in vivo induced reactivation.

Herpes simplex virus (HSV) establishes a lifelong latent infection in host sensory neurons. A hallmark of HSV latent infection is the propensity of the latent virus to reactivate at various times and produce recurrent disease. Recurrent HSV-1 corneal infection, which can lead to blindness due to scarring of the cornea, is the most common cause of infectious blindness in the developed world (18).

Although the mechanism(s) by which HSV establishes and reactivates from latency is an area of intense study, much remains unknown. The latency-associated transcript (LAT) RNAs are the only HSV RNAs that have been detected during neuronal latency (24, 30). LAT has therefore been implicated in latency. LAT produces a family of LAT RNAs including ones of 2, 1.3 to 1.5, and 8.3 kb (4, 8, 24, 27, 29, 30, 33–37, 40). The LATs overlap, in an antisense direction, two important viral genes, ICP0 and ICP34.5.

To look for a phenotype for LAT, investigators in several laboratories have made and analyzed a variety of HSV-1 LAT mutants in mice and rabbits (1, 10–12, 14, 15, 25, 28, 32). HSV-1 reactivates spontaneously in the rabbit but not in the mouse. The parental strains used to construct the published LAT mutants do not have high spontaneous reactivation rates (even in the rabbit). Thus, most of these studies have examined the effect of LAT on explant and/or induced reactivation only. Since the phenotypes for explant, induced, and spontaneous reactivation are not necessarily due to the same gene or sets of genes, none of the studies employing explant or induced reactivation have directly addressed the possible role of LAT in spontaneous in vivo reactivation. Only two studies have tried to assess spontaneous in vivo reactivation of a LAT mutant. In one study (10) a LAT mutant did not significantly decrease spontaneous reactivation. In a second study (2), in which spontaneous reactivation was reported as being significant, the authors nonetheless indicated that "The low frequency of spontaneous reactivation demonstrated by all viruses used makes meaningful investigation of the differences between experiments impossible. At this juncture, we would stress the highly significant differences in induced reactivation between LAT^+ and LAT^- viruses." Thus, the involvement of LAT in spontaneous in vivo reactivation remains to be definitively confirmed.

We report here the construction of a mutant with a LAT deletion mutation in McKrae. This HSV-1 strain has a much higher in vivo spontaneous reactivation rate than the parental strain of any previously reported LAT mutant. This LAT mutant (dLAT2903) was compared with its parental virus and with a rescued virus in which the LAT deletion has been repaired (dLAT2903R). We found that with the exception of reactivation, dLAT2903 was normal for all biological parameters examined. In contrast, spontaneous reactivation of dLAT2903 in the rabbit ocular model of HSV-1 latency and reactivation was decreased to approximately 33% of normal. This demonstrates an important role for LAT in in vivo spontaneous reactivation.

Previous studies have concluded that LAT mutants decrease but do not eliminate induced reactivation. However, these reports did not take into account the background of spontaneous reactivation. We report here that attempts to induce reactivation of dLAT2903 did not increase the reactivation rate above the background of spontaneous reactivation. Thus, we show here for the first time that (i) in an HSV-1 strain with a high spontaneous reactivation rate, deletion of LAT significantly decreased spontaneous reactivation, and (ii) deletion of LAT not only decreased but appeared to completely block induced reactivation. These highly significant differences in spontaneous ($P < 0.0001$) and induced ($P < 0.0001$) reactiva-

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tion were probably due to direct effects of LAT on reactivation, since dLAT2903 appeared to establish latency at a normal rate.

MATERIALS AND METHODS

Virus and cells. All mutants were derived from HSV-1 McKrae. The parental McKrae virus and all mutants were triple plaque purified and passaged only once or twice prior to use. Rabbit tear films were cultured on primary rabbit kidney cell monolayers to look for the presence of reactivated HSV-1. Rabbit skin (RS) cells (from Rick Thompson) were used for all other experiments, unless otherwise indicated. All cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Construction of the LAT deletion mutant dLAT2903. The previously cloned *Bam*HI B fragment of HSV-1 McKrae (22) was digested with *Eco*RV and *Hpa*I to remove a 1.8-kb fragment beginning upstream of the LAT promoter TATA box and extending 705 nucleotides into the 2-kb LAT (see the bottom of Fig. 1). *Pac*I linker (8 bp) (New England Biolabs, Beverly, Mass.) was ligated to the digested site. The *Pac*I linker containing plasmid was digested with *Pac*I restriction enzyme, purified by agarose gel electrophoresis, electroeluted from the gel, religated, and used to transform *Escherichia coli* lambdaR RICI857 (5, 22). Plasmid DNA was screened and purified by standard procedures (16). The LAT deletion mutant was made and isolated by previously published procedures (25). Briefly, the purified plasmid DNA with the 1.8-kb LAT deletion was cotransfected with purified infectious McKrae DNA into RS cells by the calcium phosphate transfection procedure. LAT deletion mutants were generated by homologous recombination. Viruses from the cotransfection were plated, and isolated plaques were picked and screened for the deletion by restriction digestion and Southern analysis. Selected plaques were triple plaque purified and reanalyzed by restriction digestion and Southern analysis to ensure that both copies of LAT contained the deletion. A final plaque was purified and designated dLAT2903. Finally, by using the same methods, the dLAT2903R rescued virus, in which the deletion was restored, was generated by cotransfection and homologous recombination of infectious dLAT2903 virus DNA with the original intact *Bam*HI-B-containing plasmid. As above, plaques were isolated from the cotransfection mix and screened by restriction digestion and Southern analysis. One plaque was triple plaque purified and reanalyzed by restriction digestion and Southern analysis to ensure that both copies of LAT had been restored.

Rabbits. New Zealand White (NZW) female rabbits, 8 to 10 weeks old (Irish Farms), were used for all experiments. Rabbits were treated in accordance with Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care, and National Institutes of Health guidelines.

Rabbit model of ocular HSV-1 infection, latency, and spontaneous reactivation. Rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of HSV-1 per eye into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s (24). At this dose of virus, virtually all of the surviving rabbits harbor a bilateral latent HSV infection in both trigeminal ganglia, resulting in a high group rate of spontaneous reactivation with HSV-1 McKrae. Latency is assumed to have been established by 28 days postinfection. Acute ocular infection of all eyes was confirmed by HSV-1 positive tear film cultures collected on days 3 and 4 postinfection.

Eye disease. The presence of eye disease was determined by examining the rabbit eyes on days 1, 3, 5, 7, 10, and 14 postinfection for herpes simplex dendritic and geographical ulceration, iritis, and conjunctivitis by slit lamp biomicroscopy with 1% fluorescein (20, 21). Each eye disease parameter was scored on a scale of 0 to 4 (0, no disease; 1, 25% involvement of the cornea; 2, 50%; 3, 75%; 4, 100%).

Ocular shedding. To test for spontaneously reactivated virus, beginning on day 30 postinfection, tear film specimens were collected daily from each eye as previously described (19), using a nylon tipped swab. The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect primary rabbit kidney cell monolayers. These cell monolayers were observed in a masked fashion by phase-light microscopy for up to 30 days for HSV-1 cytopathic effects. All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. DNA was purified from selected positive cultures derived from rabbits latently infected with the LAT deletion mutant dLAT2903 and analyzed by restriction enzyme digestion and Southern blots to confirm that the reactivated virus was identical to the input virus. This was found to be the case in all instances.

Induced reactivation. Induction was done either by iontophoresis of 6-hydroxydopamine to the cornea followed by topical application of epinephrine as we previously described (19) or by intrastromal injection of sterile saline as previously described (9). Induced ocular shedding was monitored as above.

Virus replication during acute infection. Tear films were collected as described above on various days postinfection. In experiment 1, a standard plaque assay was done with 10-fold serial dilutions of the inoculated medium. In experiment 2, the amount of virus was estimated by inoculating cells with 10-fold dilutions of the inoculated medium and scoring the results as virus positive or negative. The amount of virus in trigeminal ganglia (TGs) was similarly estimated beginning with individual disrupted TGs in 1 ml of medium.

Purification of DNA from TGs of latently infected rabbits. Individual TGs were removed from euthanized rabbits and stored at −80°C until use. To remove debris, individual TGs were transferred to sterile Eppendorf tubes containing 1.0 ml of 100% ethanol at room temperature. Each tube was vortexed for 1 min, the TG was pelleted in a microcentrifuge for 2 min and resuspended in 100% ethanol, and the procedure was repeated twice. The final pelleted TG was vacuum dried and suspended in 500 μl of Tris-EDTA (TE) containing 0.1% sodium dodecyl sulfate and 100 μg of proteinase K per ml. The mixture was incubated at 55°C for 16 h. The treated mixture was extracted once with water-saturated phenol and then with chloroform-isoamyl alcohol (24:1). The final supernatant was precipitated with 2.5 volume of 95% ethanol and vacuum dried.

Semiquantitation of latent HSV-1 DNA. Latent HSV-1 DNA was semiquantitated by PCR by the methods of Coen and coworkers (3, 13) with minor modifications. The DNA extracted from each TG was resuspended in 100 μl of double-distilled water. Half (50 μl) of the DNA was used for amplification of HSV-1 DNA, and the other half was used for amplification of rabbit actin DNA. We split the sample and ran the primer sets in parallel, because our experience is that running the primer sets together often produces anomalies. The primer set used for virus DNA amplification was from the thymidine kinase (TK) gene of HSV-1 as previously reported (31). The size of the PCR product is 110 bp. The internal probe used to detect this product is from the same study (31). The actin primer set used for amplification of cellular actin and the

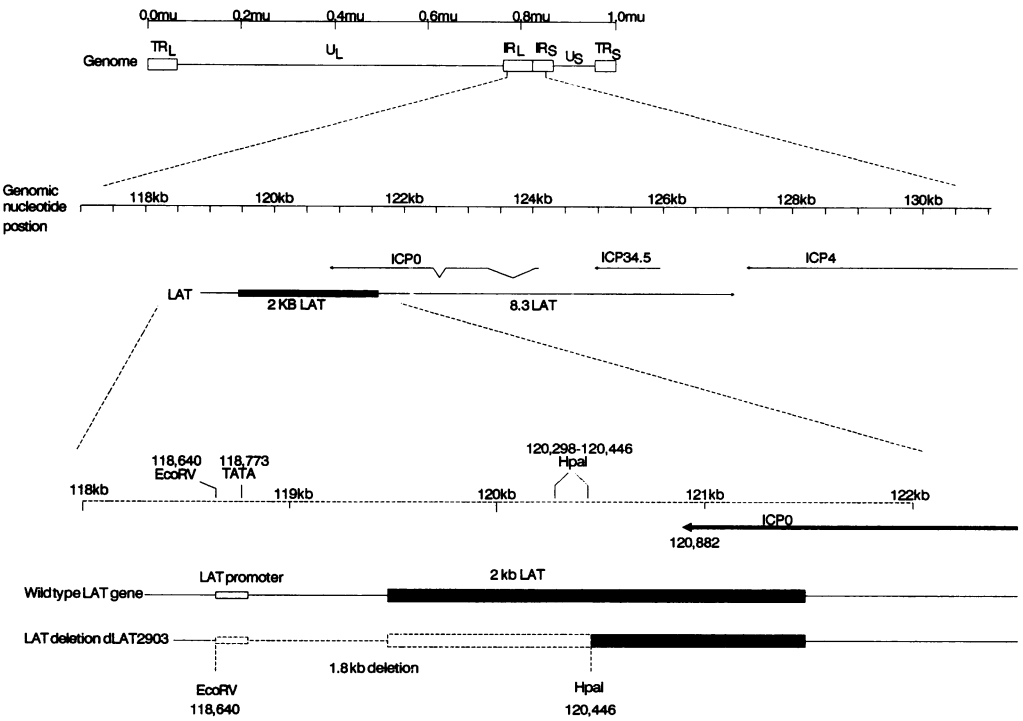

FIG. 1. Schematic representation of the LAT deletion mutant dLAT2903. The LAT gene is located in the long repeat regions (terminal long repeat and internal long repeat) of the HSV-1 genome, and the virus is thus homozygous for the LAT gene. For simplicity, only the copy of the LAT gene in the internal repeat region is shown schematically. An identical copy of LAT is also present in the terminal long repeat. The prototypic viral genomic structure with map units is shown along the top. The portion of the internal repeat region containing LAT is blown up to show the relative locations of the LAT, ICP0, gamma-34.5, and ICP4 genes. The region containing the 5′ end of LAT is further blown up to reveal additional details, including the LAT promoter region and TATA box and the locations of the *Eco*RV and *Hpa*I restriction sites that define the ends of the deletion in dLAT2903. The dashed line at the bottom of the figure indicates the location of the deletion. Nucleotide numbers are based on the complete sequence of strain 17 syn+ (17, 23).

internal probe used to detect the 124-bp PCR product were as previously reported (2).

Thirty cycles of amplification were done with *Taq* polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and the supplied buffer. Cycling reactions were performed with a thermal cycler (Appligene, Pleasanton, Calif.). Cycles were as follows: (i) denaturation at 94°C for 1 min; (ii) annealing at 5°C below the melting temperature of the primers for 1.5 min; and (iii) extension at 74°C for 2.5 min. The final cycle was terminated with a 10-min extension period at 74°C. The amplified products were fractionated on a 1.8% Sea-Kem agarose gel running in Tris-acetate-EDTA buffer. Alternate lanes contained the TK PCR product and the actin PCR product from the same TG.

The specificity of the amplified DNA products was confirmed by Southern blot hybridization of the PCR products with an internal probe. The Southern blots were exposed overnight to preflashed Kodak X-ray film at −70°C with an intensifying screen. The films were developed, and the DNA bands were scanned with a laser densitometry scanner. The relative amount of HSV-1 DNA was expressed as the ratio of the intensity of the TK PCR band to the intensity of the actin PCR band. To maintain good separation of the TK and actin PCR products (i.e., to avoid overexposed bands from infringing on lighter bands), every other lane was left empty. A twofold dilution series of a known amount of viral DNA was mixed with uninfected TGs, DNA was isolated and subjected to PCR under the same conditions, and the results were plotted on a log-log plot (see Fig. 7A). This ensured that under the conditions used the final PCR amplification product was directly related to the input amount of viral DNA in the experimental samples.

Statistical analyses. Statistical analyses were performed with Instat, a personal computer software program. For analyses by either the Student *t* test, the Mann-Whitney rank sum test, the chi-square test, or the Fisher exact test, results were considered statistically significant when $P < 0.05$. For analysis of correlations (the Spearman nonparametric correlation test), results were considered to show a correlation when the absolute $r > 0.5$ and $P < 0.05$.

RESULTS

Structure of the LAT deletion mutant dLAT2903. To examine the importance of LAT in spontaneous reactivation, we constructed a LAT deletion mutant of HSV-1 McKrae. McKrae was used as the parental virus because it has the highest spontaneous reactivation rate of any commonly used HSV-1 strain. In particular, McKrae has a much higher spontaneous reactivation rate than any of the HSV-1 strains previously used for construction of LAT mutants.

One difficulty in constructing a LAT deletion mutant is that LAT completely overlaps the ICP0 and gamma-34.5 genes (Fig. 1, center). Deletion of any of the LAT region that overlaps either of these genes would make it difficult to definitively assign to LAT any phenotypic alterations seen in

such a mutant. Therefore, our strategy was to remove the LAT promoter and as much of the 5′ LAT region as possible without infringing on any non-LAT genes.

dLAT2903 was constructed by standard homologous recombination as described in Materials and Methods. It contains a 1.8-kb deletion from nucleotides 118640 (an *Eco*RV site) to 120466 (a *Hpa*I site) (Fig. 1, bottom). This deletion extends from 138 nucleotides upstream of the LAT promoter TATA box to approximately 705 nucleotides into the 2-kb LAT and encompasses approximately 0.2 kb of the promoter and the first 1.6 kb of the primary transcript. Southern analysis of restriction enzyme digestions was used to verify that dLAT 2903 contained this deletion in both copies of LAT (data not shown). A rescued virus, dLAT2903R, in which both copies of the deleted LAT region are fully restored was made by homologous recombination of dLAT2903 with an appropriate cloned restriction fragment from McKrae as described in Materials and Methods.

As constructed, dLAT2903 is incapable of LAT transcription. It is generally accepted that the abundant 2-kb LAT and possible less-abundant LATs are all derived from a single primary LAT transcript of approximately 8.3 kb (39). This 8.3-kb LAT is transcribed under the control of the well-mapped and well-characterized LAT promoter (6, 15, 26, 37–40), which is deleted from dLAT2903. It is well documented that deletion of this LAT promoter produces viruses that make no detectable LAT-related RNAs (12). Nonetheless, the possibility of a cryptic promoter just prior to the start of the 2-kb LAT that is responsible for transcription of some of the 2-kb LAT has been raised (7). Since the deletion in dLAT2903 has removed the authentic LAT promoter, the hypothetical cryptic promoter, and over 700 nucleotides of the start of the 2-kb LAT, this mutant is unable to express either the primary 8.3-kb LAT or the 2-kb LAT. We have confirmed this by Northern (RNA) blot analysis of the RNA produced in RS cell monolayers infected with dLAT2903 or McKrae (data not shown).

Replication of dLAT2903 in tissue culture. Monolayers of RS cells were infected with dLAT2903, dLAT2903R, or McKrae at a multiplicity of infection of 0.01 or 10 PFU per cell. At various times, monolayers were harvested by freeze-thawing and virus titers were determined by plaque assay (Fig. 2). Replication of the mutant, that of the rescued mutant, and that of the parental wild type were similar at both input multiplicities at all times examined. In fact, at a multiplicity of infection of 0.01 the growth kinetics of dLAT2903 and McKrae were indistinguishable, while at a multiplicity of infection of 10 the apparently minor but insignificant differences favored the LAT deletion mutant. The similarities in growth rates of dLAT2903, dLAT2903R, and McKrae indicated that deletion of LAT did not affect viral replication in RS cell tissue culture.

Replication of dLAT2903 in vivo. To examine the ability of dLAT2903 to replicate in rabbit eyes relative to McKrae, tear films were collected on days 3, 5, and 7 postinfection from each of three rabbits (six eyes) infected with 2×10^5 PFU of McKrae per eye, 2×10^5 PFU of dLAT2903 per eye, or (in case dLAT2903 replicates poorly) 2×10^6 PFU of dLAT2903 per eye. The amount of virus in each tear film was determined by standard plaque assays (Fig. 3A). On each of the 3 days, there was no statistically significant difference in the amount of detected virus ($P > 0.05$; Mann-Whitney nonparametric test). Thus, deletion of LAT did not adversely affect the ability of HSV-1 to replicate in rabbit eyes. In fact, the tendency was for dLAT2903 to replicate better than McKrae, although, as stated above, this difference was not significant. Although the amount of virus detected in eyes infected at the higher input dose of dLAT2903 (2×10^6 PFU per eye compared with 2×10^5 PFU per eye) appeared slightly larger, this difference was also not statistically different.

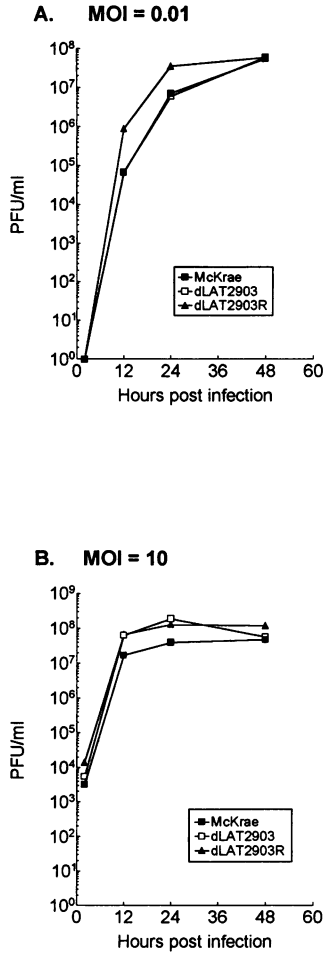

FIG. 2. Replication of dLAT2903 in tissue culture. Semiconfluent monolayers of RS cells were infected with McKrae, the LAT deletion mutant dLAT2903, or the rescued mutant dLAT2903R. Following a 1-h adsorption, the monolayers were washed with medium and refed with fresh medium containing 10% fetal calf serum. At the times indicated after infection, each monolayer was freeze-thawed twice, and the amount of total virus per milliliter was determined by plaque assays. (A) Initial infection was 0.01 PFU per cell; (B) initial infection was 10 PFU per cell. MOI, multiplicity of infection.

To compare dLAT2903 with dLAT2903R for the ability to replicate in rabbit eyes and TGs, nine rabbits (18 eyes per virus) were ocularly infected with each virus as described in Materials and Methods. On days 3, 5, and 7, three rabbits per group were sacrificed and TGs were harvested for analysis of infectious virus as described in Materials and Methods. On the same days, tears were collected from all eyes prior to sacrifice (nine rabbits per virus on day 3, six rabbits per virus on day 5, and three rabbits per virus on day 7). The amount of virus was estimated by analyzing 10-fold dilutions for the ability to produce cytopathic effects (Fig. 3B). As in the above comparison of dLAT2903 and McKrae, on each day the average amount of virus detected per eye was not different for the LAT deletion mutant and the rescued virus ($P > 0.3$ for each day; Student t test). The average amount of infectious virus detected in TGs (Fig. 3C) was also not different on these days for

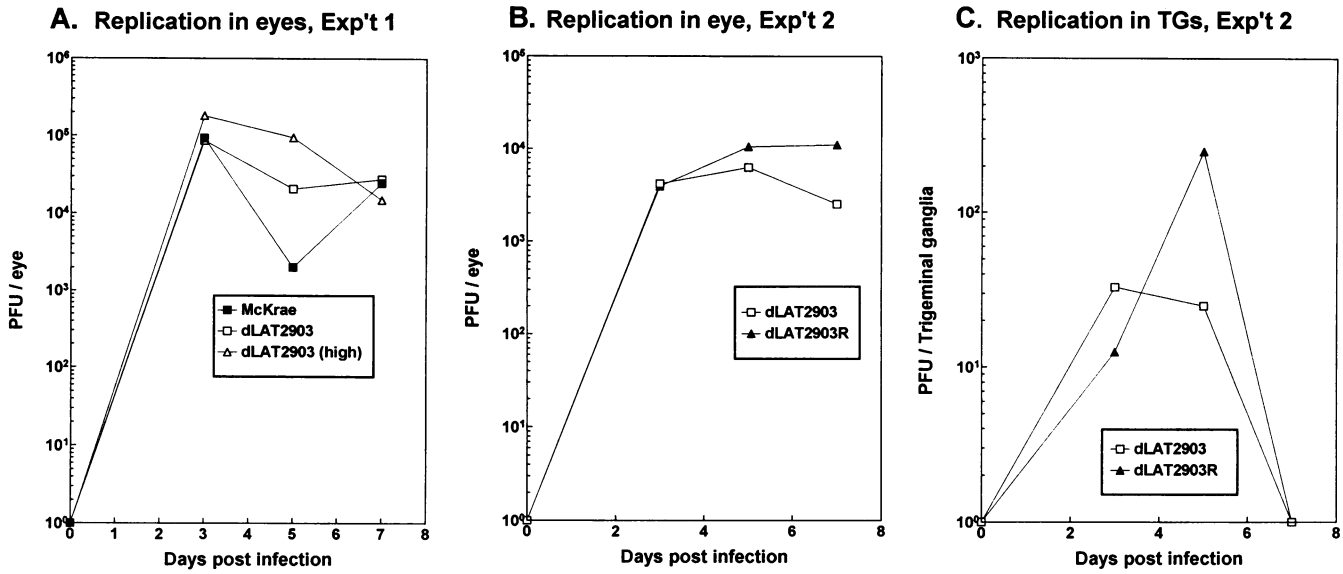

FIG. 3. Replication of dLAT2903 in rabbit eyes and TGs. (A) Rabbits were infected with 2×10^5 PFU of McKrae per eye, 2×10^5 PFU of the LAT deletion mutant dLAT2903 per eye, or 2×10^6 PFU of dLAT2903 per eye, as described in Materials and Methods. At 2 h postinfection (day 0) and on days 3, 5, and 7 postinfection, tear films were collected as described in Materials and Methods, and the amount of infectious virus per eye was determined by plaque assays. The average of titers from six eyes for each virus at each time point are shown. (B) Nine rabbits per group were infected with dLAT2903 or dLAT2903R (2×10^5 PFU per eye), tear films were collected as above, and 10-fold serial dilutions were analyzed for the ability to produce cytopathic effect. The average results are shown for 18 eyes per virus on day 3, 12 eyes per virus on day 5, and 6 eyes per virus on day 7. (C) Six rabbits per group from panel B were sacrificed on days 3, 5, and 7. TGs were disrupted, and 10-fold serial dilutions were analyzed as above. The average results for six TGs per virus per time point are shown.

dLAT2903 and dLAT2903R (six ganglia per group per day; $P > 0.1$ for each day). Thus, deletion of LAT did not appear to alter acute HSV-1 replication in the rabbit eye or the rabbit TGs. Therefore, LAT did not appear to play a significant role in replication of HSV-1 in the eye, spread of HSV-1 to the TGs, or replication of HSV-1 in the TGs.

Eye disease induced by the LAT deletion mutant. Infection of rabbit eyes with HSV-1 McKrae produces ocular disease similar to that seen in humans. Conjunctivitis, dendritic ulceration, geographical ulceration, and clouding were measured during acute infection of 18 eyes infected with the LAT deletion mutant and 18 eyes infected with the parental McKrae virus. These measurements were done daily by slit-lamp biomicroscopy as described in Materials and Methods. The average peak eye disease in each group is shown in Fig. 4. No statistically significant differences were seen between dLAT2903 and McKrae infection for any of the eye disease parameters ($P > 0.05$ for all parameters; Student t test). Thus, deletion of LAT had no significant effect on eye disease. This suggests that LAT does not play an important role in eye disease in the rabbit.

Neurovirulence of dLAT2903. Neurovirulence and spontaneous and induced reactivation of dLAT2903 in bilaterally ocularly infected rabbits were analyzed in vivo in two independent experiments. In experiment 1, rabbits were infected with dLAT2903 or McKrae. There was no statistically significant difference in the survival rates between McKrae (4 of 9, 44%) and dLAT2903 (11 of 18, 61%; $P = 0.47$, Fisher exact test, two sided) (Table 1).

In experiment 2, rabbits were infected with dLAT2903 or dLAT2903R. The survival rates for the LAT deletion mutant and the LAT rescued viruses were identical (12 of 20, 60%; $P = 1.25$) (Table 1). This rate was also similar to that seen in experiment 1 for McKrae ($P = 1.0$). Thus, deletion of LAT did not appear to alter survival, suggesting that LAT does not play a critical role in neurovirulence of HSV-1 in the rabbit.

Spontaneous reactivation of the LAT deletion mutant. Rabbit eyes were infected with dLAT2903, McKrae, or dLAT 2903R. Beginning 30 days postinfection (at which time latency had already been established), all eyes were swabbed once a

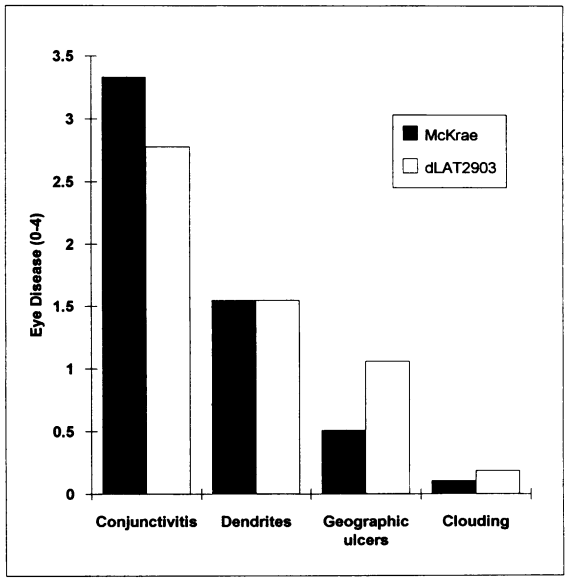

FIG. 4. Eye disease in rabbits infected with dLAT2903. Rabbits were bilaterally ocularly infected with McKrae or dLAT2903 as described in Materials and Methods. Disease was scored on a scale of 0 to 4 (no disease to 100% corneal involvement) on days 3, 5, 7, 10, 14, and 21 as described in Materials and Methods. The average peak scores for 18 eyes per group for each disease parameter are shown.

TABLE 1. Effect of a LAT deletion on survival and spontaneous and induced reactivation[a]

Expt	No. of survivors/ total no. (%)	No. of spontaneous reactivation-positive cultures/ total no. (%)	No. of spontaneous reactivation-positive eyes/total no. (%)	No. of spontaneous reactivation episodes/eye	No. of eyes induced to reactivate/total no. (%)
Expt 1					
dLAT2903	11/18 (61)	21/484 (4)	9/22 (41)	0.5 (n = 22)	0/22 (0)
McKrae	4/9 (44)	27/176 (15)	7/8 (88)	1.7 (n = 8)	3/8 (38)
P (dLAT2903 vs McKrae)	0.47[b]	<0.0001[b]	0.04[b]	0.02[c]	0.01[b]
Expt 2					
dLAT2903	12/20 (60)	24/816 (3)	9/24 (38)	0.7 (n = 24)	1/24 (4)
dLAT2903R	12/20 (60)	62/816 (8)	18/24 (75)	1.1 (n = 24)	10/24 (42)
P (dLAT2903 vs dLAT2903R)	1.25	<0.0001[b]	0.02[b]	0.02[c]	0.004[b]

[a] Rabbits were bilaterally ocularly infected with dLAT2903 (LAT$^-$), McKrae (LAT$^+$), or dLAT2903R (LAT$^+$) viruses as described in Materials and Methods. The infectious dose of all viruses was 2×10^5 PFU per eye except for one-half of the dLAT2903 infected rabbits in experiment 1, which were infected with 2×10^6 PFU per eye. No significant differences in results were seen between these doses, and the results were pooled for convenience. Survival was assessed 21 days postinfection. Spontaneous reactivation was assessed beginning on day 28 postinfection (22 days, experiment 1; 34 days, experiment 2) as described in Materials and Methods. Reactivations were induced by iontophoresis or by intracorneal injection as described in Materials and Methods.

[b] Fisher exact test, two sided. The groups are considered significantly different if $P < 0.05$.

[c] Mann-Whitney rank sum test, two sided. The groups are considered significantly different if $P < 0.05$.

day to collect tear films for analysis of reactivated virus as described in Materials and Methods. Experiment 1 compared the LAT deletion mutant dLAT2903 with the parental McKrae virus. Experiment 2 compared dLAT2903 with the rescued dLAT2903R virus.

The cumulative number of virus-positive tear film cultures during 22 days (experiment 1) or 34 days (experiment 2) is shown in Fig. 5. Because of the different number of rabbits (and eyes) in the two experiments and between the LAT-positive and LAT-negative viruses in experiment 1, the data were standardized to represent cumulative positive cultures per eye. The cumulative spontaneous reactivation rate in rabbits latently infected with dLAT2903 appeared greatly reduced compared with that in McKrae-infected rabbits (Fig. 5A) or dLAT2903R-infected rabbits (Fig. 5B).

A statistical analysis of positive (spontaneously reactivated) cultures versus negative cultures is shown in Table 1, column 3. In experiment 1, 15% (27 of 176) of the McKrae cultures contained spontaneously reactivated virus. In contrast, only 4% (21 of 484, 3.75-fold fewer) of the tear films from eyes infected with the LAT deletion mutant were positive for reactivated virus. This difference was highly significant ($P <$

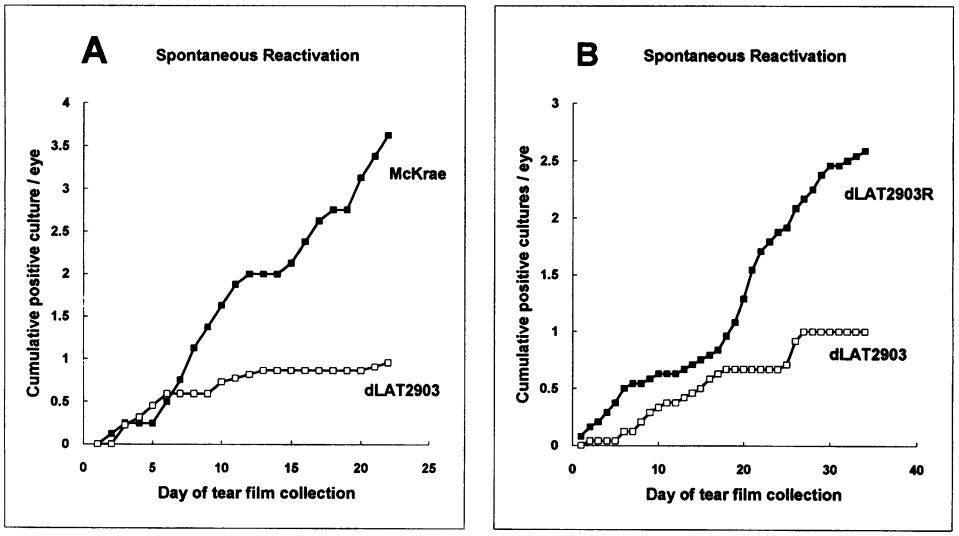

FIG. 5. Cumulative spontaneous reactivation in rabbits infected with the LAT deletion mutant, dLAT2903. Rabbits were bilaterally ocularly infected with 2×10^5 PFU of either McKrae, dLAT2903, or dLAT2903R per eye as described in Materials and Methods. Tear films were collected daily for 22 days (A) or 34 days (B) beginning on day 30 postinfection (day 1). The tear films were plated on indicator cells and observed for up to 30 days for the presence of spontaneously reactivated virus as described in Materials and Methods. The results are plotted as the cumulative number of virus-positive cultures divided by the total number of cultures divided by the number of eyes. This gives the cumulative number of the average positive tear film cultures per eye on each day. At the end of each experiment, the dLAT2903-infected eyes averaged approximately one positive culture each. The McKrae (A)-infected eyes averaged over three positive cultures each, and the dLAT2903R (B)-infected eyes averaged almost three positive cultures each. The total number of cultures and positive cultures is shown in Table 1, column 3. (A) Experiment 1. Solid squares represent the results from 16 eyes latently infected with McKrae; open squares represent the results from 22 eyes latently infected with dLAT2903. (B) Experiment 2. Solid squares, 24 McKrae eyes; open squares, 24 dLAT2903R eyes.

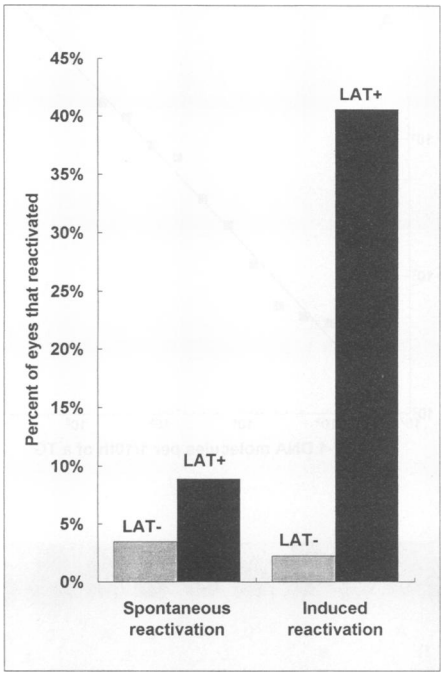

FIG. 6. Summary of spontaneous and induced reactivation of the LAT deletion mutant dLAT2903. The results of experiments 1 and 2 were pooled for the LAT$^-$ virus (dLAT2903) and the LAT$^+$ viruses (McKrae plus dLAT2903R). The combined results for spontaneous reactivation (Table 1, column 3) and induced reactivation (Table 1, column 6) are shown. Combined spontaneous reactivation: LAT$^-$ virus, 45 of 1,300 cultures, 3.5%; LAT$^+$ viruses, 89 of 992 cultures, 9% ($P < 0.0001$, Fisher exact test). Combined induced reactivation: LAT$^-$ virus, 1 of 46 eyes, 2%; LAT$^+$ viruses, 13 of 32 eyes, 41% ($P < 0.0001$).

0.0001). Similar results were obtained in experiment 2. dLAT 2903 had a spontaneous reactivation rate of only 3% (24 of 816), almost threefold less than with dLAT2903R (8%, 62 of 816). As in experiment 1, this difference was highly significant ($P < 0.0001$) (combined results shown graphically in Fig. 6, left side). Thus, deletion of LAT produced a pronounced decrease in spontaneous reactivation. This suggests that LAT is an important factor in spontaneous reactivation in the rabbit eye model.

Because the above analyses do not take into account the number of eyes in each of the groups, the data were analyzed by additional methods. The fraction of virus-positive cultures for each eye in each group (i.e., the fraction of time each eye was virus positive) was calculated, and these fractions were analyzed by the Student t test. In both experiments, the decreased spontaneous reactivation rate of the LAT deletion mutant compared with the parental or rescued virus was statistically significant (McKrae versus dLAT2903, $P = 0.021$; dLAT2903R versus dLAT2903, $P = 0.015$).

The number of eyes in each group that had at least one spontaneous reactivation is shown in Table 1, column 4. In experiment 1, 88% (7 of 8) of the McKrae infected eyes reactivated spontaneously while only 41% (9 of 22) of the dLAT2903-infected eyes reactivated spontaneously ($P = 0.04$). In experiment 2, 75% (18 of 24) of the eyes infected with the rescued LAT mutant dLAT2903R reactivated spontaneously, compared with only 38% (9 of 24) of the eyes infected with the LAT deletion mutant. Again, this difference was statistically significant ($P = 0.02$).

Another method of examining the effect of the LAT deletion on spontaneous reactivation is to analyze the number of times that spontaneous reactivation is detected in each eye, regardless of the length of time that virus is present. This is equivalent to the number of episodes in which reactivated virus is detected in the tears, with consecutive days of positive cultures being treated as a single event. Thus, an eye that sheds virus for a single day and an eye that sheds virus for five consecutive days would both constitute one episode of spontaneous reactivation. The average number of spontaneous reactivations per eye in each group calculated in this manner is shown in Table 1, column 5. In experiment 1, the number of spontaneous reactivations in the dLAT2903-infected eyes averaged 0.5 compared with 1.7 for McKrae-infected eyes ($P = 0.02$). In experiment 2, dLAT2903-infected eyes averaged 0.7 spontaneous reactivation compared with 1.1 episodes per eye for dLAT2903R-infected eyes ($P = 0.02$). All of the above analyses strongly support the notion that LAT plays a significant and important role in spontaneous reactivation in the rabbit eye model.

Induced in vivo reactivation. Following the period of tear film collection for analysis of spontaneous reactivations, in vivo reactivations were induced by iontophoresis of 6-hydroxydopamine followed by topical epinephrine (experiment 1) or injection of sterile water between the layers of the cornea (experiment 2) (see Materials and Methods). In vivo induced reactivation was scored positive only if tear films on day 3 or 4 (or both) following induction were positive for virus (Table 1, column 6). Approximately 40% of the eyes infected with the parental McKrae virus (3 of 8) or the rescued LAT deletion mutant (10 of 24) were induced to reactivate. In contrast, none of the dLAT2903-infected eyes were induced to reactivate in experiment 1 (0 of 22; $P = 0.01$) and only one of the dLAT2903-infected eyes appeared to be induced to reactivate in experiment 2 (1 of 24, 4%; $P = 0.004$) (combined results shown graphically in Fig. 6, right side). In experiment 2, tear films were collected for two additional days. No additional virus-positive eyes were detected on day 5 or 6 postinduction in dLAT2903-infected rabbits. Thus, the lack of induced reactivation on days 3 and 4 postinduction was not due to a slight delay in the induced reactivation of dLAT2903.

The single dLAT2903 virus-positive eye was virus positive on both days 3 and 4 postinduction. Thus, of the 48 cultures assayed on these two days in experiment 2 and the 44 cultures assayed on these two days in experiment 1, approximately 2% (2 of 92) were virus positive. Since, as determined above, the spontaneous reactivation rate for dLAT2903 was 3 to 4%, the 2% reactivation rate detected following induction was not above the background spontaneous reactivation rate expected for this mutant virus during this period. Thus, the single reactivation event seen with dLAT2903 may have been the result of spontaneous rather than induced reactivation. These results suggest that the LAT deletion mutant responded very poorly (if at all) to stimuli capable of inducing reactivation of wild-type (and rescued) virus.

Rate of establishment of latency. Deletion of LAT might result in a reduced rate of establishing latency, and this could account for the apparent reduced reactivation rates seen with dLAT2903. To address this, at the termination of experiment 2 the rabbits were sacrificed and both TGs were removed. Total DNA was isolated from each TG, and the relative amounts of HSV-1-specific DNA were determined for each TG by semiquantitative PCR analysis as described in Materials and Methods. Two sets of PCR primers were used. One set was specific for a region of the HSV-1 TK gene. The other set was specific for a portion of cellular actin. This was used as an internal

control to standardize the recovery of DNA from the TGs and to standardize any differences in efficiency of DNA transfer during Southern blotting. The relative intensity of each PCR product band following Southern analysis was determined by laser scanning as described in Materials and Methods, and the ratio of each HSV-1 specific band and the corresponding actin band was calculated. This number represents the standardized relative amount of HSV-1 DNA.

To ensure that the PCR was linear over the range of HSV-1 DNA present in the samples and also to produce a standard curve from which the amount of HSV-1 DNA in the latent TGs could be extrapolated, a control experiment was run in parallel. Twofold serial dilutions containing known amounts of HSV-1 genomic DNA in the range of approximately 10^3 to 10^6 copies were added to test tubes each containing a control TG from uninfected rabbits. The total DNA was then isolated and subjected to PCR by the same procedures used for the DNA from the latently infected rabbits. The autoradiographic intensities of the HSV-1 PCR products of the serially diluted DNA were plotted on a log-log scale versus the known amount of input HSV-1 DNA (Fig. 7A). The result was a relatively straight line, indicating that in this range of HSV-1 DNA our PCR assay was quantitative.

An autoradiogram of a typical set of experimental PCRs is shown in Fig. 7B. Visually, the intensity of viral DNA (D) from the dLAT2903R rescued virus (R) and the dLAT2903 LAT deletion virus (L) appeared similar, relative to the intensity of their corresponding actin (A) DNA. Following laser densitometry scanning of this and similar autoradiograms, the amount of HSV-1 DNA relative to actin from 24 individual TGs from the dLAT2903R-infected rabbits was calculated to be 1.82 ± 0.45 (Table 2, column 2). The relative amount of HSV-1 DNA in the 24 individual TGs from the dLAT2903 mutant was 2.28 ± 0.69. Although the LAT deletion mutant-infected TGs appeared to contain an average of 27% more latent HSV-1 DNA than the rescued virus, this difference was not statistically significant ($P = 0.62$). These results strongly suggest that the dLAT2903 LAT deletion mutant was not defective in its ability to establish latency. Thus, following ocular infection, LAT does not appear to play an important role in the establishment of latency in rabbit TGs. These results therefore also indicate that the decreased spontaneous and induced reactivation rates seen with the LAT deletion mutant dLAT2903 were not due to a decreased ability of this virus to establish latency. Thus, the notion that LAT plays a direct role in the in vivo reactivation process in the rabbit is strongly supported.

As judged by extrapolation to the standard plot in Fig. 7A, the amount of experimental DNA PCR products corresponded to averages of approximately 8×10^5 and 1×10^6 copies of viral DNA per TG in the rabbits latently infected with dLAT2903R and dLAT2903, respectively (Table 2, column 3). In the rabbits latently infected with dLAT2903R, the number of HSV-1 genomic copies varied from approximately 4.0×10^4 to 4.4×10^6 per TG, with one DNA-negative TG (<1,000 copies). In the rabbits latently infected with dLAT2903, the number of HSV-1 genomic copies varied from approximately 5.2×10^4 to 7.4×10^6, with two DNA-negative TGs. This approximately 100-fold range of values is similar to that reported for the number of copies of HSV-1 DNA in TGs of latently infected mice (13).

Lack of correlation between the relative amount of latent HSV-1 DNA in each TG and the observed spontaneous and induced reactivation rates for the corresponding eye. The analyses described above made it possible to look for correlations between the amount of HSV-1 DNA in each TG (a measure of latency) and the amount of spontaneous or induced reactivation in the corresponding eye. Although searches for correlations on a group basis have been previously attempted (25), to our knowledge this was the first attempt to look for correlations between individual sets of TGs and eyes. A Spearman nonparametric correlation test was done with the data for the TGs and eyes from rabbits latently infected with dLAT2903R or dLAT2903. An absolute $r > 0.5$ and $P < 0.05$ would indicate a correlation. No correlation was found between the relative amount of latent HSV-1 DNA and spontaneous or induced reactivations in either group (Table 2, columns 4 and 5) (absolute $r < 0.3$, $P > 0.2$). Thus, at the limits of the current levels of sensitivity, we were unable to detect any

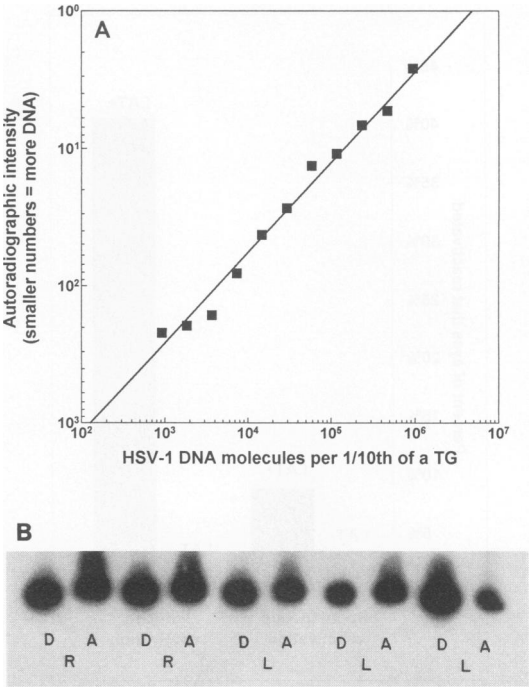

FIG. 7. Southern blot analysis of the PCR products from total DNA isolated from latently infected TGs. (A) As a control standard, twofold serial dilutions of HSV-1 genomic DNA representing approximately 10^6 to 10^3 molecules were each mixed with a TG from an uninfected rabbit and total DNA was isolated and subjected to the identical PCR protocols used for panel B. The autoradiographic intensity of an internal TK probe, used to determine the amount of HSV-1 TK PCR product, was plotted against the copy number of input genomic DNA on a log-log graph. The computer program used to analyze the intensity of the autoradiographic bands reports small numbers for dark areas and large numbers for light areas. Thus, increasing PCR product goes from the bottom to the top of the *y* axis. (B) At the conclusion of experiment 2, TGs were harvested from sacrificed rabbits. As described in Materials and Methods, total DNA was individually isolated from each TG and aliquots were subjected to PCR with primer pairs specific for either the HSV-1 TK gene or the gene for cellular actin. The PCR products were then separated by agarose electrophoresis, blotted, hybridized to an internal probe for the TK product and the actin product, and exposed to X-ray film for autoradiography. Adjacent lanes contain the TK and actin PCR products from the same TGs. The results from these and similar blots (24 dLAT2903 and 24 dLAT2903R DNA samples) were quantitated, and the ratio of the intensity of hybridization to the TK and actin products was determined. By using this normalized intensity of hybridization of the TK PCR product, the amount of DNA in each TG was also estimated by extrapolation to the plot in panel A. The results are shown in Table 2.

TABLE 2. Relative and semiquantitative amounts of latent HSV-1 DNA in rabbit TGs and analysis of correlations between HSV-1 DNA and reactivation in the corresponding eye[a]

Virus	Relative amt of HSV-1 DNA in TGs[b,c]	Range (mean) of HSV-1 genome copies in latently infected TGs[d]	Correlation: relative amt of HSV-1 DNA with spontaneous reactivation	Correlation: relative amt of HSV-1 DNA with induced reactivation
dLAT2903	2.28 ± 0.69 (n = 24)	5.2×10^4–7.4×10^6 (1×10^6)	$r = -0.05$, $P = 0.81$[e]	$r = -0.09$, $P = 0.69$[e]
dLAT2903R	1.82 ± 0.45 (n = 24)	4.0×10^4–4.4×10^6 (8×10^5)	$r = -0.23$, $P = 0.28$	$r = -0.09$, $P = 0.76$

[a] The relative amount of HSV-1 DNA in individual TGs from rabbits latently infected with dLAT2903 or dLAT2903R was determined as described in Materials and Methods.

[b] Mean ± standard error.

[c] P (dLAT2903 vs dLAT2903R) = 0.62 (Student t test, two sided).

[d] The estimated amount of DNA was quantitated for each of 24 latently infected TGs per virus by extrapolation of the normalized intensity of the PCR products to the graph shown in Fig. 7A.

[e] Spearman nonparametric correlation test. An absolute $r > 0.5$ combined with a $P < 0.05$ would indicate a correlation. These results show no correlation.

correlation between the level of ganglionic latency and reactivation.

DISCUSSION

All previous HSV-1 LAT mutants have been constructed in virus strains that do not have high in vivo spontaneous reactivation rates. Studies to examine the role of LAT in reactivation from latency have therefore almost exclusively used in vitro explant reactivation models or in vivo induced reactivation models. Thus, there is little direct information regarding the potential role of LAT in in vivo spontaneous reactivation. To overcome this, we have constructed a LAT deletion mutant in HSV-1 McKrae and studied the effect of this mutation on in vivo spontaneous reactivation in the rabbit ocular model of HSV-1 latency and reactivation. McKrae was chosen as the parental virus because it has the highest spontaneous reactivation rate of any of the commonly used HSV-1 laboratory strains.

Since LAT overlaps at least two important HSV-1 genes, ICP0 and ICP34.5, a deletion of the entire LAT gene cannot be used to examine the role of LAT in spontaneous reactivation. We therefore constructed dLAT2903 to contain a large LAT promoter deletion and the largest possible deletion of the 5′ end of LAT, while ensuring that no other known HSV-1 genes were disturbed. This was done for two reasons. First, to ensure that no LAT transcription could occur from the LAT promoter, we deleted the entire region containing the LAT promoter rather than just deleting or altering a small region assumed to be crucial, such as the TATA box. Second, to ensure that no transcription of the 2-kb LAT could occur from a potential cryptic promoter just upstream of the 2-kb LAT, the deletion also covers this potential cryptic 2-kb LAT promoter and the first third of the 2-kb LAT itself. Thus, dLAT2903 is incapable of transcribing any LAT from the bona fide LAT promoter or from the potential cryptic promoter. This was confirmed by Northern blots. Other, smaller LAT promoter deletion mutants have previously been shown to produce no detectable LAT RNA in tissue culture or during neuronal latency (12, 14, 28).

dLAT2903 was compared with the parental McKrae virus and with the rescued virus, dLAT2903R. Except for differences in reactivation, the LAT deletion mutant was indistinguishable from the parental and rescued viruses. The LAT deletion mutant replicated in tissue culture with the same kinetics and to the same final titer as wild-type virus, regardless of the initial multiplicity of infection. Likewise, replication of dLAT2903 in rabbit eyes and rabbit TGs was the same as that of wild-type virus. The rate at which dLAT2903 established latency was also indistinguishable from that of the wild-type viruses. In contrast, dLAT2903 was significantly restricted in its ability to reactivate spontaneously.

In the experiments reported here, the spontaneous reactivation rate for McKrae was 15%, compared with 8% for the rescued LAT mutant dLAT2903R. We have found that such variation in spontaneous reactivation rates from experiment to experiment is common, even with the same virus stock. These variations are thought to be due to variations in variables such as the age and health of the groups of rabbits used at different times and the fact that New Zealand White rabbits are not an inbred strain. Thus, the spontaneous reactivation rates of 8% for dLAT2903R and 15% for McKrae are both consistent with our previous McKrae studies. In fact, substitution of the results for dLAT2903R in place of McKrae in experiment 1 still indicated a statistically significant decrease in the spontaneous reactivation rate of dLAT2903 ($P < 0.05$, Fisher exact test). Thus, the higher spontaneous reactivation rate seen in experiment 1 with McKrae did not alter the conclusion that in experiment 1 (as well as in experiment 2) the LAT deletion mutant had a reduced spontaneous reactivation rate.

The rate of in vivo spontaneous reactivation for dLAT2903 averaged approximately one-third that of the parental or rescued virus (spontaneous reactivation rates combined from both experiments: dLAT2903, 3%; McKrae plus dLAT2903R, 9%). Thus, deletion of LAT significantly reduced but did not eliminate spontaneous reactivation. This suggests that there may be two categories of spontaneous reactivations, LAT-dependent spontaneous reactivations (which represent approximately two-thirds of the spontaneous reactivations in this system) and LAT-independent spontaneous reactivations (which represent approximately one-third of the spontaneous reactivations in this system).

In contrast to the one-third residual spontaneous reactivation seen with dLAT2903, our results suggest that induced in vivo reactivation in dLAT2903 may have been eliminated rather than reduced. Only 2% of eyes (1 of 46) from rabbits latently infected with dLAT2903 showed reactivation following in vivo induction, compared with 41% of eyes (13 of 32) from rabbits latently infected with dLAT2903R and McKrae. Induced reactivation was measured on days 3 and 4 postinduction, and the single dLAT2903-reactivating eye was positive on both days. Since all other eyes were negative on both days, the "induced" shedding rate was 2 of 92 (2%). This rate is likely to have been due to the spontaneous reactivation of dLAT2903 (3 to 4%). Thus, deletion of the LAT promoter may have rendered dLAT2903 refractory to induced in vivo reactivation.

On the basis of the above discussion, we propose the following possibility: (i) in the rabbit ocular model of HSV-1 latency and reactivation, LAT is required for in vivo induction of reactivation; (ii) approximately two-thirds of spontaneous

reactivations are LAT dependent and may therefore represent in vivo induced reactivations in which the inducing factor(s) is not elucidated; and (iii) approximately one-third of spontaneous reactivations are independent of LAT and represent a low level of background spontaneous reactivations.

Regardless of whether the above speculation is correct or whether LAT simply increases the probability of a spontaneous reactivation event by threefold, LAT appears to be a major factor in both in vivo induced reactivations and classic spontaneous reactivations. Assuming that the mechanisms of spontaneous and induced reactivations in humans are accurately reflected in the rabbit model, our results strongly suggest that LAT is a crucial factor in clinically important HSV-1 reactivations.

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