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The Latency-Associated Transcript Gene Enhances Establishment of Herpes Simplex Virus Type 1 Latency in Rabbits

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The latency-associated transcript (LAT) gene the only herpes simplex virus type 1 (HSV-1) gene abundantly transcribed during neuronal latency, is essential for efficient in vivo reactivation. Whether LAT increases reactivation by a direct effect on the reactivation process or whether it does so by increasing the establishment of latency, thereby making more latently infected neurons available for reactivation, is unclear. In mice, LAT-negative mutants appear to establish latency in fewer neurons than does wild-type HSV-1. However, this has not been confirmed in the rabbit, and the role of LAT in the establishment of latency remains controversial. To pursue this question, we inserted the gene for the enhanced green fluorescent protein (EGFP) under control of the LAT promoter in a LAT-negative virus (ΔLAT-EGFP) and in a LAT-positive virus (LAT-EGFP). Sixty days after ocular infection, trigeminal ganglia (TG) were removed from the latently infected rabbits, sectioned, and examined by fluorescence microscopy. EGFP was detected in significantly more LAT-EGFP-infected neurons than ΔLAT-EGFP-infected neurons (4.9% versus 2%, $P < 0.0001$). The percentages of EGFP-positive neurons per TG ranged from 0 to 4.6 for ΔLAT-EGFP and from 2.5 to 11.1 for LAT-EGFP ($P = 0.003$). Thus, LAT appeared to increase neuronal latency in rabbit TG by an average of two- to threefold. These results suggest that LAT enhances the establishment of latency in rabbits and that this may be one of the mechanisms by which LAT enhances spontaneous reactivation. These results do not rule out additional LAT functions that may be involved in maintenance of latency and/or reactivation from latency.

Following primary ocular infection, herpes simplex virus type 1 (HSV-1) establishes a lifelong latent infection in sensory neurons of the trigeminal ganglia (TG). At various times throughout the life of the infected individual, the virus can reactivate, return to the eye, be shed in tears, and produce recurrent corneal disease and scarring leading to impaired vision. Recurrent ocular HSV-1 results in over 400,000 doctor visits per year in the United States and is a leading infectious cause of corneal blindness (15).

During latency, abundant viral transcription is consistently detected only in the region of the latency-associated transcript (LAT) gene (25, 30). LAT is located in the long repeats of the HSV-1 genome and is therefore present in two copies per genome. The primary LAT transcript is 8.3 kb (33, 34). 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 (4). LAT null mutants (i.e., LAT transcription-negative mutants) have been shown to reactivate poorly by explant or induced reactivation in the mouse (9, 10, 28, 29), by induced reactivation in the rabbit (8, 32), and by spontaneous reactivation in the rabbit (16, 19).

The molecular mechanisms by which LAT enhances reactivation are not understood. It is also not known (i) whether LAT functions to enhance the establishment of latency, thereby increasing reactivation by providing more latently infected neurons, (ii) whether LAT functions solely at the level of reactivation, or (iii) whether LAT functions both in establishment and reactivation. LAT may also be involved in maintenance of latency. LAT is antisense to and completely overlaps the important immediate early gene ICP0 (25, 30, 34). It was therefore proposed that in neurons LAT might suppress ICP0 by an antisense mechanism and that this might be involved in the establishment and maintenance of latency (25, 30). However, we have shown that a LAT mutant capable of expressing only the first 1.5 kb of LAT, a region that does not overlap IPC0, has normal, wild-type (wt) levels of spontaneous reactivation in the rabbit (19). This suggests that LAT's main function is not that of antisense down regulation of ICP0.

Most studies directed at determining whether LAT-negative (LAT^-) mutants establish reduced levels of latency in rabbits have been inconclusive. Estimating the amount of latent HSV-1 DNA in the TG of latently infected rabbits has not revealed significant differences between LAT^- and LAT-positive (LAT^+) viruses (1, 16). This may be due to the high variability in the amount of latent HSV-1 DNA present in different TG within each group. PCR analysis of DNA from individual neurons isolated from mouse TG showed that mice latently infected with LAT^- HSV-1 had fewer positive neurons than mice latently infected with LAT^+ HSV-1 (26, 27, 31). In addition, during latency in mice, a LAT^+ virus expressing β-galactosidase produced more β-galactosidase-positive neurons than a LAT^- virus expressing β-galactosidase (28). Other studies have shown that during acute (5) and latent (2) infection in the mouse, LAT suppresses expression of other viral genes. The above mouse studies strongly suggest that LAT is involved in either the establishment or the maintenance of HSV-1 latency in mice. Similar studies using rabbits have not been reported.

For reasons not fully understood, latent HSV-1 DNA cannot be detected by in situ hybridization of sections from latently infected TG from mice, rabbits, or humans. Recently, in situ

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PCR has been used to detect latent HSV-1 DNA and quantitate the number of HSV-1 DNA-positive neurons in sections of mouse TG. More neurons contained HSV-1 DNA in mice latently infected with LAT$^+$ virus compared to mice latently infected with LAT$^-$ virus (12, 31). As above, similar studies remain to be performed in the rabbit.

Since LAT is the only HSV-1 gene abundantly transcribed during latency (25, 30), the percent of neurons in the TG containing LAT RNA has been used as a relative measure of the amount of latency (18). This method has been used to estimate the effect on latency of mutants other than LAT. Obviously, this method cannot be used to compare LAT$^-$ viruses to LAT$^+$ viruses, since LAT$^-$ viruses make no LAT. However, it may be possible to make use of the fact that the percentage of neurons containing LAT is a reflection of the percentage of neurons in which the LAT promoter is highly active. We therefore constructed a LAT$^+$ and a LAT$^-$ virus, each expressing the enhanced green fluorescent gene (EGFP) under control of the LAT promoter. We report here that during latency in rabbits, more neurons in the TG were positive for EGFP with the LAT$^+$ virus than with the LAT$^-$ virus. These results suggest that as in the mouse, in the rabbit LAT plays a role in either the establishment and/or the maintenance of neuronal latency in TG.

MATERIALS AND METHODS

Virus and cells. All mutants were derived from HSV-1 strain McKrae. The parental McKrae virus and all mutants were triple plaque purified and passaged only one or two times prior to use. Rabbit skin (RS) cells grown in Eagle's minimal essential media supplemented with 10% fetal calf serum were used for all experiments.

Construction of viral mutants. The parental virus for ΔLAT-EGFP was *d*LAT2903, a mutant of HSV-1 strain McKrae containing a 1.8-kb (*Eco*RV/*Hpa*I) deletion in both copies of LAT that removed 0.2 kb of the LAT promoter and 1.6 kb of the 5′ end of the primary 8.3-kb LAT transcript (LAT nucleotides −161 to +1667) in both copies of LAT (16). ΔLAT-EGFP (Fig. 1C) was constructed by homologous recombination between *d*LAT2903 DNA and a plasmid containing the entire structural gene for EGFP, using previously published methods (16, 17, 20). To make this plasmid, EGFP [including a 3′ poly(A) signal; Clontech, Palo Alto, Calif.] was subcloned into a plasmid such that the final construct contained EGFP flanked by regions of LAT contained in *d*LAT2903 (LAT nucleotides −798 to +76 and 1667 to 1850). The resulting ΔLAT-EGFP virus contains two copies of EGFP, one in each long repeat, under transcriptional control of the LAT promoter. LAT-EGFP (Fig. 1D) was constructed from ΔLAT-EGFP by inserting a 3.3-kb restriction fragment comprising the LAT promoter and the first 1.5 kb of the primary LAT transcript exactly as previously described for inserting this restriction fragment into *d*LAT2903 (19). LAT-EGFP-2 (Fig. 1F) was constructed from LAT3.3A (Fig. 1E) by inserting the EGFP gene into the LAT deletion in both long repeats as described above for the construction of ΔLAT-EGFP.

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

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 into the conjunctival cul-de-sac of each eye, closing the eye, and rubbing the lid gently against the eye for 30 s (28). At this dose of HSV-1 McKrae, virtually all of the surviving rabbits harbor a bilateral latent HSV infection in both TG, resulting in a high group rate of spontaneous reactivation with the McKrae strain of HSV-1. 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 day 3 or 4 postinfection.

Replication of virus in vivo. Rabbits were infected as described above. As previously described (16), on various days postinfection tear films were collected by eye swab, the swabs were placed in tissue culture media, and the amount of virus was determined by plaque assay.

Detection and quantitation of EGFP. One TG from each rabbit was removed at autopsy, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight, and mounted in OCT compound (Electron Microscopy Sciences, Washington, Pa.), and 5- to 6-μm sections were cut with a Leica CM1850 cryostat (Leica Microsystems, Nussloch, Germany). Each TG was positioned prior to sectioning such that all sections had similar cross sections and contained neurons from all three regions of the TG. As many sections as possible were cut from each TG (approximately 300), and then approximately 10 sections/TG representative of the entire TG (e.g., sections 25, 50, 100, 125, 150, 175, 200, 225, 250, and 275) were mounted on slides. Sections containing fewer than 100 neurons (because of tissue loss) were not used. An average of just over six usable sections were obtained from each TG. The sections were directly examined by fluorescence microscopy (model BX-40 equipped with a video camera computer-controlled imaging capture system; Olympus, Melville, N.Y.). The filter set used was 41012-703 HQ:FLP (Chroma Technology Corp., Brattleboro, Vt.). With this filter set, EGFP-positive cells appeared traffic light green (bright green with a hint of blue), and very little background was seen. In addition, the background was greenish yellow, a color distinctly different, and readily differentiated, from the bright green-blue color of EGFP fluorescence. Prior attempts to use standard fluorescein isothiocyanate filters resulted in very high background and the inability to readily distinguish EGFP-positive cells from the background of EGFP-negative cells, as background and EGFP fluorescence were both an apple green color.

Neutralizing antibody titers. As previously described (22), 50 PFU of wt HSV-1 was incubated for 30 min at 37°C with twofold serial dilutions of individual rabbit sera, plated in triplicate on monolayers of RS cells in 12-well plates, overlaid with medium containing 1% methylcellulose, incubated for 3 days at 37°C, and stained with crystal violet; then plaques were counted. The 50% plaque reduction titer for each individual sera was calculated using the formula $PDD_{50} = DL + \{(P_{50} - PL)(DH - DL)/(PH - PL)\}$, where *DL* is the reciprocal of the lower dilution bracketing the 50% endpoint, *PL* is the number of plaques at the lower dilution bracketing the 50% endpoint, *DH* is the reciprocal of the higher dilution bracketing the 50% endpoint, *PH* is the number of plaques at the higher dilution bracketing the 50% endpoint, and P_{50} is the number of plaques at the 50% endpoint. To ensure that anti-EGFP antibody did not interfere with HSV-1 infections or determinations of HSV-1 neutralizing antibody titers, HSV-1 neutralizing antibody titers were determined using commercially available anti-EGFP antibody (Clontech). No neutralizing activity was detected (data not shown).

ELISA titers against EGFP. Enzyme-linked immunosorbent assays (ELISAs) were performed on sera as previously described (6, 14), using purified EGFP (Clontech) as the capture antigen. Briefly, 96-well plates were coated with 50 μl of EGFP, incubated overnight at 4°C, washed with PBS-Tween 20 (0.3%), incubated with threefold serial dilutions of serum for 1 h at 37°C, washed with PBS-Tween 20, incubated with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G–alkaline phosphatase for 1 h at 37°C, and incubated with 50 μl of Sigma 104 phosphatase substrate for 30 min at 25°C; 50 μl of 3 N NaOH was added to stop the reaction, and plates were read on an ELISA reader at 405 nm. Titers are expressed as the reciprocal of the dilution having an absolute reading of 0.1. Rabbits infected with LAT-EGFP or ΔLAT-EGFP developed similar very high ELISA antibody titers against EGFP (>1:16,000 [data not shown]).

Statistical analyses. Statistical analyses were performed using Prizm GraphPad, a personal computer software program. Results were considered statistically significant when the *P* value was <0.05.

RESULTS

Construction of EGFP expressing viruses. ΔLAT-EGFP contains EGFP under control of the LAT promoter in a LAT$^-$ virus (Fig. 1C). ΔLAT-EGFP was constructed by inserting the LAT promoter driving EGFP into the normal LAT location (one in each long repeat) of *d*LAT2903 (16), a LAT null mutant (Fig. 1B). The second virus, LAT-EGFP, contains EGFP under control of the LAT promoter in a LAT$^+$ virus (Fig. 1D). LAT-EGFP was constructed by inserting a 3.3-kb restriction fragment containing the LAT promoter and the first 1.5 kb of LAT into the unique long region of ΔLAT-EGFP between the genes UL37 and UL38. We previously showed that inserting the same restriction fragment into the same location in *d*LAT2903, the LAT null mutant from which ΔLAT-EGFP was constructed, completely restored wt levels of spontaneous reactivation (19). Thus, the only difference between ΔLAT-EGFP and LAT-EGFP should be that LAT-EGFP contains a functional LAT. Additional details of the construction of these mutants is presented in Materials and Methods.

EGFP-positive neurons in TG of rabbits latently infected with LAT-EGFP and ΔLAT-EGFP. Rabbits were bilaterally ocularly infected with LAT-EGFP or ΔLAT-EGFP (2×10^5 PFU/eye) without scarification as described in Materials and Methods (16, 23). Sixty days postinfection, a time at which latency was well established, rabbits were euthanized, and the TG were removed. The TG were sectioned and examined by

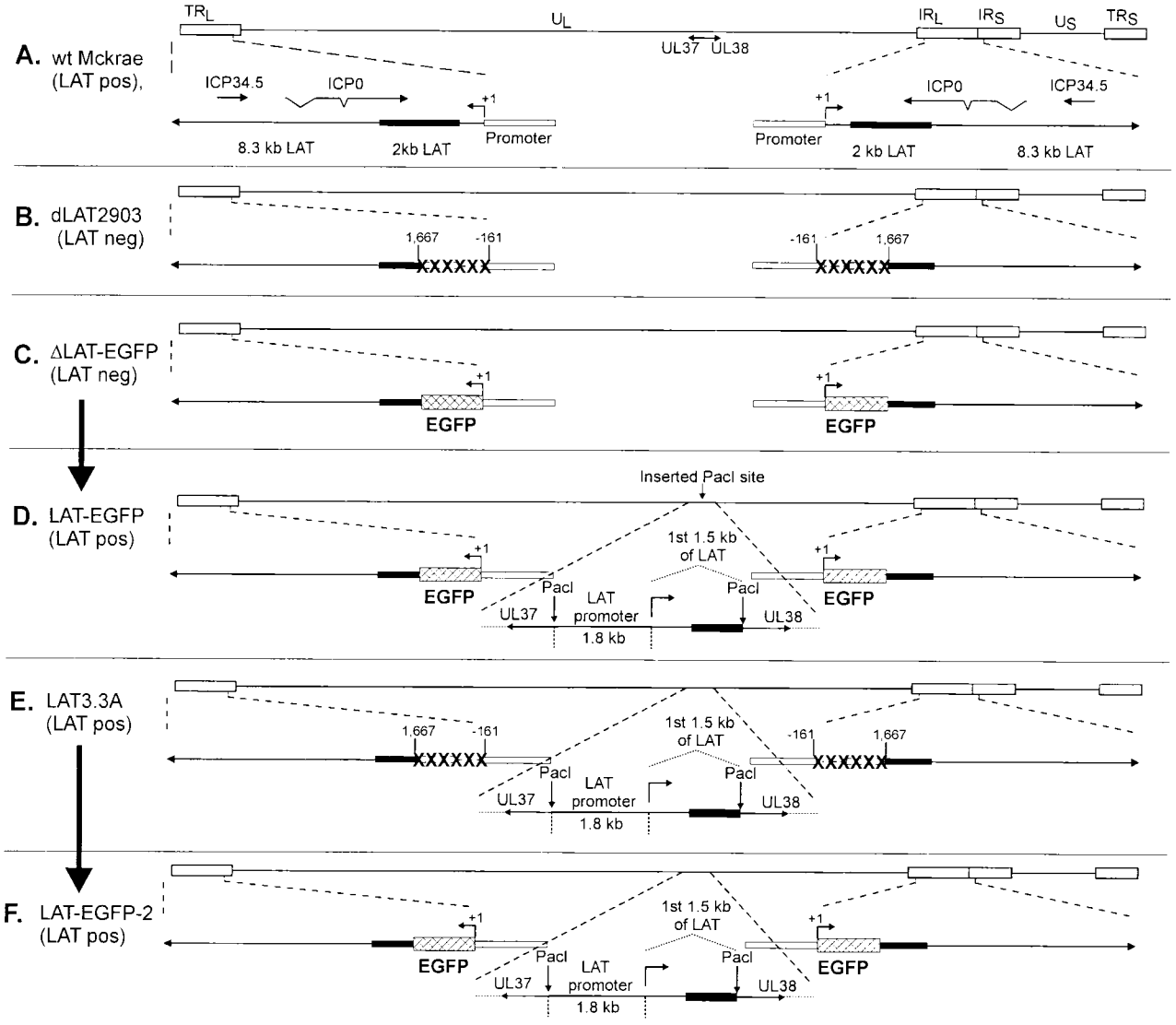

FIG. 1. Structure of LAT-EGFP and ΔLAT-EGFP viruses. (A) Structure of the HSV-1 McKrae genome in the prototypic orientation. The open rectangles represent the repeat regions of the virus (TRL, terminal repeat long; IRL, internal repeat long; IRS, internal repeat short; TRS, terminal repeat short) that bound the unique long (UL) and unique short (US) regions. The long repeats are expanded to show more detailed structure of the LAT region (one in each long repeat). The largest arrow represents the location of the primary LAT. Locations of the ICP0 and ICP34.5 transcripts are shown for reference. The solid rectangle represents the very stable 2-kb LAT. The start of LAT transcription is indicated by the arrow at +1. (B) *d*LAT2903 has a deletion from LAT nucleotides −161 to +1667 in both copies of LAT, makes no LAT RNA, and reactivates poorly. We have previously described the construction and properties of *d*LAT2903 (16). (C) ΔLAT-EGFP was constructed from *d*LAT2903 by homologous recombination between *d*LAT2903 DNA and a plasmid containing the complete LAT promoter and the entire structural EGFP gene [including a 3′ poly(A) signal] flanked by regions of LAT contained in *d*LAT2903 (LAT nucleotides −798 to +76 and 1667 to 1850) as described in Materials and Methods. The resulting virus contains two copies of EGFP, one in each long repeat, under transcriptional control of the LAT promoter. (D) LAT-EGFP was derived from ΔLAT-EGFP (vertical arrow) by insertion by homologous recombination of a 3.3-kb restriction fragment comprising the LAT promoter and the first 1.5 kb of LAT in an ectopic location between UL37 and UL38. We previously showed that insertion of this 3.3-kb LAT fragment into this location completely restored wt levels of spontaneous reactivation to the LAT null mutant *d*LAT2903 (19). (E) LAT3.3A (previously designated LAT1.5a) (19) is deleted for LAT in both long repeats, contains the 3.3-kb LAT ectopic insert described in panel D, and has wt levels of spontaneous reactivation. (F) LAT-EGFP-2 was constructed from LAT3.3A (vertical arrow) by insertion of the LAT promoter and the entire structural EGFP gene [including a 3′ poly(A) signal] as described for panel C. The final structures of LAT-EGFP and LAT-EGFP-2 should be identical. The structures of all the viruses were confirmed by restriction enzyme digestion and Southern analyses.

fluorescence microscopy using a filter set optimized for the detection of EGFP fluorescence as described in Materials and Methods. Representative images are shown in Fig. 2. EGFP-positive neurons (arrows) appear a bright greenish blue that is easily distinguished from the yellow-green background. EGFP-positive neurons appeared more numerous in TG from rabbits latently infected with the LAT^+ LAT-EGFP virus (Fig. 2A) compared to TG from rabbits latently infected with the LAT^- ΔLAT-EGFP virus (Fig. 2B). No EGFP-positive neurons were seen in control TG from uninfected rabbits (Fig. 2C).

Quantitation of EGFP-positive neurons in TG of rabbits latently infected with LAT-EGFP and ΔLAT-EGFP. The number of EGFP-positive neurons and the total number of neurons were determined on individual sections from 19 different TG (8 LAT-EGFP and 11 ΔLAT-EGFP), each from a different rabbit. An average of six sections were examined from each TG (see Materials and Methods). Over 30,000 neurons were evaluated for EGFP expression (Fig. 3A). Of the neurons from rabbits latently infected with LAT-EGFP, 4.9% were positive for EGFP. In contrast, only 2% of the neurons from rabbits

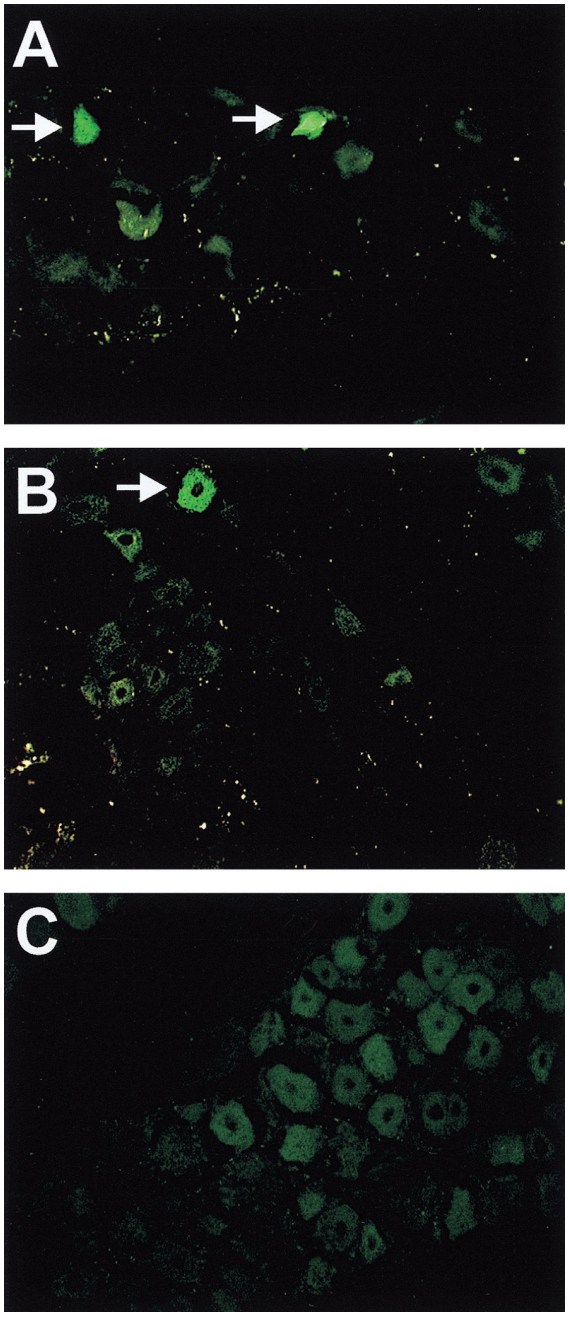

FIG. 2. Detection of EGFP in TG of rabbits latently infected with LAT-EGFP and ΔLAT-EGFP. Rabbits were bilaterally ocularly infected with the HSV-1 McKrae strain-derived mutant LAT-EGFP or ΔLAT-EGFP (2×10^5 PFU/eye) without corneal scarification as described in Materials and Methods. Sixty days postinfection, the rabbits were euthanized and the TG were removed. As described in Materials and Methods, frozen TG sections were prepared and the presence of EGFP was determined, without additional manipulation, by immunofluorescence microscopy. Representative results are shown. (A) TG from a rabbit latently infected with LAT-EGFP; (B) TG from a rabbit latently infected with ΔLAT-EGFP; (C) TG from an uninfected rabbit. Panel C is slightly overexposed compared to panels A and B to confirm the lack of EGFP fluorescence. The arrows indicate neurons that were scored positive for EGFP.

latently infected with ΔLAT-EGFP were positive for EGFP. This difference was highly significant ($P < 0.0001$; chi square). No obvious difference was seen in the intensity of the EGFP fluorescence in neurons from ΔLAT-EGFP compared to LAT-EGFP latently infected rabbits.

The percentage of EGFP-positive neurons per TG is shown in Fig. 3B. All eight of the TG from rabbits latently infected with LAT-EGFP contained EGFP-positive neurons, with the percentage of EGFP-positive neurons per TG ranging from 2.5 to 11.1. In rabbits latently infected with ΔLAT-EGFP, the percentage of EGFP-positive neurons per TG ranged from only 0 to 4.6 $P = 0.003$). Furthermore, while all 8 TG from the LAT^+ infected rabbits had at least 2.5% EGFP-positive neurons, only 2 of the 11 TG from LAT^- infected rabbits contained more than 2.5% EGFP-positive neurons. Taken together, the above results suggest that LAT increased the number of neurons expressing EGFP an average of two- to threefold.

The sections used above should be representative of the TG from which they were derived since, as described in Materials and Methods, they were systematically chosen to span the entire TG. To confirm that most of the sections from a given TG had similar percentages of EGFP-positive neurons, the percentages of EGFP-positive neurons for all sections in each individual TG were plotted as a scattergram (Fig. 4). For each TG, most of the sections contained similar percentages of EGFP-positive neurons, indicating that for the majority of the TG there was not a great deal of scatter among the sections. This confirmed that as regards the percentage of EGFP-positive neurons, the sections examined were representative of the TG from which they were cut.

Replication of LAT-EGFP and ΔLAT-EGFP in rabbit eyes. To ensure that the above results were not due to differential replication of the two mutants, we analyzed replication of LAT-EGFP and ΔLAT-EGFP. Replication of LAT-EGFP and that of ΔLAT-EGFP were similar in rabbit eyes (Fig. 5).

Spontaneous reactivation of LAT-EGFP and ΔLAT-EGFP. We recently showed that serum neutralizing antibody titers in

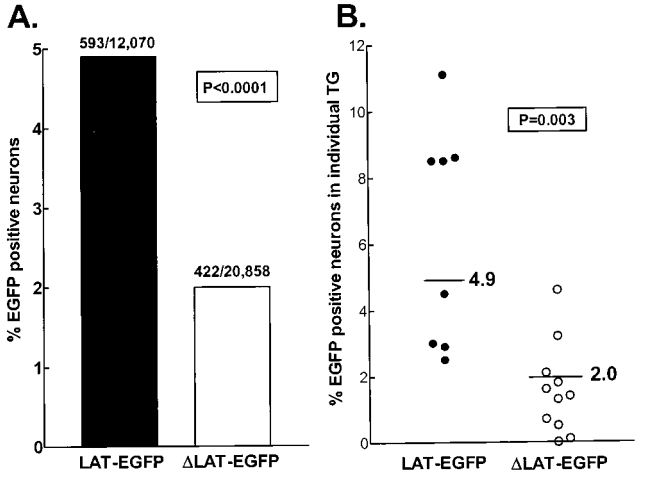

FIG. 3. Quantitation of EGFP-positive neurons in TG of rabbits latently infected with LAT-EGFP or ΔLAT-EGFP. (A) Total EGFP-positive neurons. TG from rabbits latently infected with LAT-EGFP (one TG from each of 8 rabbits) or ΔLAT-EGFP (one TG from each of 11 rabbits) were sectioned and examined for EGFP-positive neurons as described in Materials and Methods. In the ΔLAT-EGFP group, 20,858 neurons were examined for EGFP on 68 sections from 11 TG (average of 6.2 sections/TG). In the LAT-EGFP group, 12,070 neurons were examined for EGFP on 49 sections from 8 TG (average of 6.1 sections/TG). *P* for the number of EGFP-positive neurons in TG from LAT-EGFP versus ΔLAT-EGFP latently infected rabbits was determined by the Student *t* test. (B) Percentage of EGFP-positive neurons per TG. Each point represents the percentage of EGFP-positive neurons in a single TG. The horizontal bars indicate the means for each group. *P* was determined by the Mann-Whitney rank sum test.

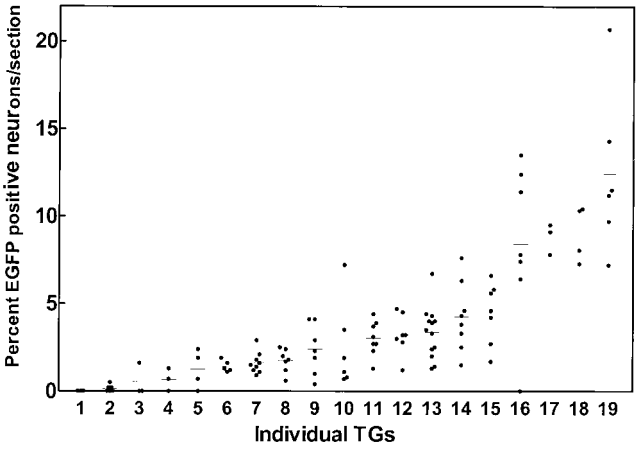

FIG. 4. Distribution of EGFP-positive neurons in sections from individual TG. The percentage of EGFP-positive neurons in each of the sections from the 19 TG in Fig. 3 is shown. Each point represents the percentage of EGFP-positive neurons on a single section. Each vertical collection of points represents a single TG. The horizontal bars indicate the mean for each TG. For ease of comparison, the TG are arranged in order from the lowest to the highest percentage of EGFP-positive neurons.

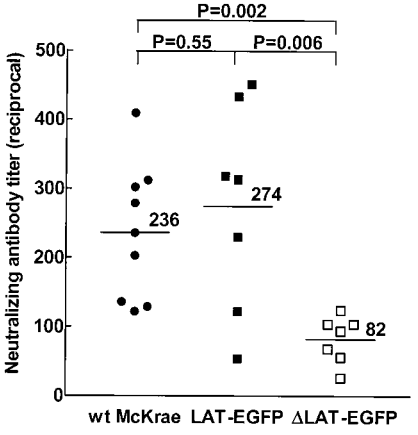

FIG. 6. Serum neutralizing antibody in rabbits 60 days postinfection. Rabbits were infected as described for Fig. 5. Serum was collected 60 days postinfection (>30 after latency had been established). HSV-1 neutralizing antibody titers were determined on individual serum samples as described in Materials and Methods. Each point represents the reciprocal of the neutralizing antibody titer for one serum sample. The horizontal bars and numbers show the mean neutralizing antibody titer. *P* values were determined by the Student *t* test.

rabbits infected with wt spontaneously reactivating viruses are two- to threefold higher on days 59 to 80 postinfection than neutralizing antibody titers in rabbits infected with viruses with poor spontaneous reactivation, and that this can be used to assess spontaneous reactivation (22). Rabbits were infected with wt McKrae, LAT-EGFP, or ΔLAT-EGFP (2×10^5 PFU/eye). Serum was collected 60 days post infection, and HSV-1 neutralizing antibody titers were determined on individual sera as described in Materials and Methods. The average neutralizing antibody titer for LAT-EGFP was similar to that for wt virus (Fig. 6). In contrast, the average neutralizing antibody titer for ΔLAT-EGFP was significantly less than that for either LAT-EGFP or wt McKrae. These results strongly suggest that, as expected, LAT-EGFP has a spontaneous reactivation rate similar to that of wt virus while ΔLAT-EGFP has a low spontaneous reactivation rate similar to those of other LAT null mutants.

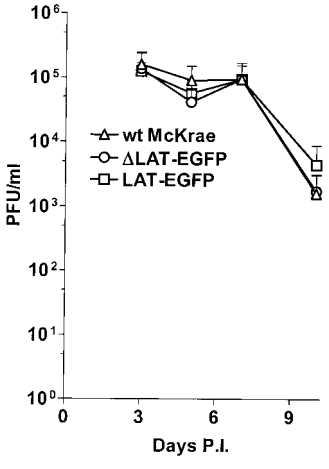

FIG. 5. Replication of LAT-EGFP and ΔLAT-EGFP in rabbit eyes. Rabbits were ocularly infected, and tear films were collected at the times shown as described in Materials and Methods. The amount of virus recovered from individual eyes was determined by plaque assays on RS cells. Each point is the mean ± standard deviation of five eyes (one per rabbit). P.I., postinfection.

DISCUSSION

The results reported here suggest that in rabbits, LAT increased the number of latently infected neurons expressing EGFP an average of two- to threefold. This in turn suggests that the LAT^+ virus, LAT-EGFP, established latency in two- to threefold more neurons than the LAT^- virus ΔLAT-EGFP and that LAT therefore increased the establishment of latency in rabbit TG two- to threefold. This conclusion is consistent with that obtained in mice latently infected with LAT^+ versus LAT^- viruses expressing β-galactosidase (28). It is also consistent with results obtained by single-cell PCR analysis of TG from mice latently infected with LAT^+ versus LAT^- viruses (31). Thus, it appears that LAT enhances the establishment (and/or maintenance) of latency in both mice and rabbits.

If, following spontaneous reactivation, HSV-1 infected and established latency in previously uninfected neurons, it could be argued that greater reactivation by LAT-EGFP (LAT^+) virus might lead to more latently infected neurons. The increased number of EGFP-positive neurons seen on day 60 postinfection in the LAT-EGFP latently infected rabbits compared to the ΔLAT-EGFP (LAT^-) latently infected rabbits might then be due to increased reactivation of the LAT^+ virus rather than an initial higher rate at which latency was established. However, this was not the case. In rabbits latently infected with either HSV-1 McKrae (the strain used here) or HSV-1 17syn+, the percentage of LAT-expressing neurons in the TG remains constant between days 20 and 360 postinfection (7). Thus, the number of LAT^+ neurons, and hence the number of LAT-EGFP-positive neurons, does not increase over time, and the percentage of EGFP-positive neurons appears to be a direct function of the initial amount of latency established.

Since LAT-EGFP was LAT^+ and ΔLAT-EGFP was LAT^-, it was expected that LAT-EGFP would have a spontaneous reactivation rate similar to that of the parental wt HSV-1 strain McKrae and that ΔLAT-EGFP would reactivate poorly. Surprisingly, using the standard method of plating rabbit tear films on indicator cells to detect spontaneously reactivated virus (16), spontaneous reactivation appeared low with both viruses (data not shown). Also surprisingly, although corneal scarring in rabbits is usually infrequent, both EGFP viruses caused

corneal scarring in the majority of rabbit eyes. Corneal scarring interferes with the detection of both spontaneous and in vivo induced reactivation in rabbit tears, and rabbit eyes with corneal scarring are therefore not included in reactivation assays (unpublished results and Jim Hill, personal communication).

Although the difficulty in detecting spontaneous reactivation of LAT-EGFP was probably due to interference by corneal scarring, it was also possible that it might be due to an unexpected mutation during construction of the virus. In particular, there may have been incorrect homologous recombination during insertion of the LAT promoter and the first 1.5 kb of LAT into the ectopic location between UL37 and UL38. This could have produced a virus that did not express the proper RNA to restore spontaneous reactivation. Therefore, we constructed a second LAT-EGFP mutant using an alternative approach. This mutant, designated LAT-EGFP-2, was constructed beginning with LAT3.3A (Fig. 1E) (previously designated LAT1.5a) (19). LAT3.3A already contains the LAT promoter and the first 1.5 kb of the primary LAT transcript in the ectopic location between UL37 and UL38, and it has wt spontaneous reactivation despite lacking LAT in the normal location in both long repeats (19). We then inserted EGFP driven by the LAT promoter into the normal LAT location in both long repeats (Fig. 1F). The amount of spontaneous reactivation detected in tears of rabbits latently infected with LAT-EGFP-2 was similar to that for LAT-EGFP (data not shown). Since LAT-EGFP and LAT-EGFP-2 were independently constructed using different approaches, this result suggests that our inability to detect high levels of spontaneous reactivation in rabbit tears was a result of some property of these viruses, probably the high level of corneal scarring. However, as judged by neutralizing antibody titers (Fig. 6), and as predicted based on their LAT^+ and LAT^- genotypes, LAT-EGFP was wt for spontaneous reactivation while ΔLAT-EGFP had reduced spontaneous reactivation.

Whether the 2- to 3-fold increase in the establishment of latency that LAT appears to mediate accounts completely (or even partially) for LAT's ability to increase spontaneous reactivation 2- to 10-fold (3, 11, 16, 19, 21) remains to be determined. We believe it likely that LAT also functions directly in the reactivation process and that LAT probably has multiple functions, some of which are difficult to assess in animal models but may have clinical significance. This would help explain the large size of the LAT gene and why the first 1.5 kb (or less than 20%) of LAT is sufficient for wt levels of spontaneous reactivation in the rabbit model of ocular HSV-1 (19).

Interestingly, the number of EGFP-positive neurons in the LAT^+ latently infected rabbits (4.9%) was similar to the number of neurons containing the stable 2-kb LAT in wt latently infected rabbits as detected by in situ hybridization of sections of TG (25, 34). This observation suggests that in both instances we are detecting neurons in which the LAT promoter is highly active. Furthermore, it suggests that the 2% EGFP-positive neurons seen in LAT^- ΔLAT-EGFP latently infected rabbits represents that percentage of neurons in which the LAT promoter is highly active in the absence of the entire LAT gene. This inference supports the notion that the percentage of EGFP-positive neurons can be used as a relative measure of the establishment of latency in LAT^+ LAT-EGFP compared to LAT^- ΔLAT-EGFP.

In situ PCR suggests that 20 to 40% of neurons in TG of latently infected mice contain HSV-1 DNA (12, 13, 24, 31). Thus, the percentage of neurons with highly active LAT promoter activity may be an underestimate of the actual percentage of latently infected neurons. In situ RT-PCR for LAT RNA detects approximately three- to fivefold more LAT-containing neurons than does standard in situ hybridization (13, 24). Since in situ reverse transcription-PCR is theoretically capable of detecting many fewer LAT copies than standard in situ hybridization, this suggests that LAT-positive neurons detected by in situ hybridization represent neurons in which the LAT promoter is highly active and that in situ reverse transcription-PCR detects neurons containing both highly active and less active LAT promoters. Whether neurons with highly active LAT promoters or neurons with less active LAT promoters (or no LAT promoter activity) are more or less likely to reactivate is unknown. Nonetheless, it remains highly likely that the percentage of neurons with high LAT promoter activity, as judged by the detection of LAT RNA by in situ hybridization or the detection of EGFP in rabbits infected with LAT-EGFP or ΔLAT-EGFP, is a reliable method of determining relative levels of HSV-1 neuronal latency. In addition, the ability of the LAT promoter EGFP constructs to produce long-term, high levels of EGFP in neurons suggests that the LAT promoter may be useful in gene therapy applications in neurons.

It is of interest that as shown in Fig. 3, there appeared to be a small amount of overlap in the amount of latency in individual TG between the LAT^+ and LAT^- groups. Thus, 2 of 11 TG in the LAT^- group appeared to contain as many latently infected neurons as 3 to 4 of the 8 TG in the LAT^+ group. This may explain why when rabbits are infected with LAT null mutants, spontaneous reactivation still occurs in some TG, although the overall spontaneous reactivation level is significantly decreased (16).

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REFERENCES

1. **Bloom, D. C., G. B. Devi-Rao, J. M. Hill, J. G. Stevens, and E. K. Wagner.** 1994. Molecular analysis of herpes simplex virus type 1 during epinephrine-induced reactivation of latently infected rabbits in vivo. J. Virol. **68:**1283–1292.
2. **Chen, S. H., M. F. Kramer, P. A. Schaffer, and D. M. Coen.** 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. **71:**5878–5884.
3. **Drolet, B., G. Perng, R. Villosis, S. Slanina, A. Nesburn, and S. Wechsler.** 1999. Expression of the first 811 nucleotides of the HSV-1 latency associated transcript (LAT) partially restores wild type spontaneous reactivation to a LAT null mutant. Virology **253:**96–106.
4. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. USA **88:**790–794.
5. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J. Virol. **71:**5885–5893.
6. **Ghiasi, H., A. B. Nesburn, R. Kaiwar, and S. L. Wechsler.** 1991. Immunoselection of recombinant baculoviruses expressing high levels of biologically active herpes simplex virus type 1 glycoprotein D. Arch. Virol. **121:**163–178.
7. **Hill, J. M., B. M. Gebhardt, R. Wen, A. M. Bouterie, H. W. Thompson, R. J. O'Callaghan, W. P. Halford, and H. E. Kaufman.** 1996. Quantitation of herpes simplex virus type 1 DNA and latency-associated transcripts in rabbit trigeminal ganglia demonstrates a stable reservoir of viral nucleic acids during latency. J. Virol. **70:**3137–3141.
8. **Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology **174:**117–125.
9. **Leib, D. A., C. L. Bogard, M. Kosz-Vnenchak, K. A. Hicks, D. M. Coen, D. M. Knipe, and P. A. Schaffer.** 1989. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63:**2893–2900.
10. **Leib, D. A., K. C. Nadeau, S. A. Rundle, and P. A. Schaffer.** 1991. The promoter of the latency-associated transcripts of herpes simplex virus type 1

contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency. Proc. Natl. Acad. Sci. USA **88:**48–52.
11. **Loutsch, J. M., G. C. Perng, J. M. Hill, X. Zheng, M. E. Marquart, T. M. Block, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. Identical 371-base-pair deletion mutations in the LAT genes of herpes simplex virus type 1 McKrae and 17syn+ result in different in vivo reactivation phenotypes. J. Virol. **73:**767–771.
12. **Maggioncalda, J., A. Mehta, Y. H. Su, N. W. Fraser, and T. M. Block.** 1996. Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia. Virology **225:**72–81.
13. **Mehta, A., J. Maggioncalda, O. Bagasra, S. Thikkavarapu, P. Saikumari, T. Valyi-Nagy, N. W. Fraser, and T. M. Block.** 1995. In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. Virology **206:**633–640.
14. **Mertz, G. J., R. Ashley, R. L. Burke, J. Benedetti, C. Critchlow, C. C. Jones, and L. Corey.** 1990. Double-blind, placebo-controlled trial of a herpes simplex virus type 2 glycoprotein vaccine in persons at high risk for genital herpes infection. J. Infect. Dis. **161:**653–660.
15. **Nesburn, A. B. (ed.).** 1983. Report of the corneal disease panel: vision research: a national plan 1983–1987, vol. II, part III. The C. V. Mosby Co., St. Louis, Mo.
16. **Perng, G. C., E. C. Dunkel, P. A. Geary, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1994. 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. J. Virol. **68:**8045–8055.
17. **Perng, G. C., H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1994. An improved method for cloning portions of the repeat regions of herpes simplex virus type 1. J. Virol. Methods **46:**111–116.
18. **Perng, G. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1996. High-dose ocular infection with a herpes simplex virus type 1 ICP34.5 deletion mutant produces no corneal disease or neurovirulence yet results in wild-type levels of spontaneous reactivation. J. Virol. **70:**2883–2893.
19. **Perng, G. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1996. The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript. J. Virol. **70:**976–984.
20. **Perng, G. C., S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1996. A 371-nucleotide region between the herpes simplex virus type 1 (HSV-1) LAT promoter and the 2-kilobase LAT is not essential for efficient spontaneous reactivation of latent HSV-1. J. Virol. **70:**2014–2018.
21. **Perng, G. C., S. M. Slanina, A. Yuhkt, B. S. Drolet, W. J. Keleher, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. A herpes simplex virus type 1 latency-associated transcript mutant with increased virulence and reduced spontaneous reactivation. J. Virol. **73:**920–929.
22. **Perng, G. C., S. M. Slanina, A. Yuhkt, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1999. Herpes simplex virus type 1 serum neutralizing antibody titers increase during latency in rabbits latently infected with latency-associated transcript (LAT)-positive but not LAT-negative viruses. J. Virol. **73:** 9669–9672.
23. **Perng, G. C., R. L. Thompson, N. M. Sawtell, W. E. Taylor, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1995. An avirulent ICP34.5 deletion mutant of herpes simplex virus type 1 is capable of in vivo spontaneous reactivation. J. Virol. **69:**3033–3041.
24. **Ramakrishnan, R., P. L. Poliani, M. Levine, J. C. Glorioso, and D. J. Fink.** 1996. Detection of herpes simplex virus type 1 latency-associated transcript expression in trigeminal ganglia by in situ reverse transcriptase PCR. J. Virol. **70:**6519–6523.
25. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61:**3820–3826.
26. **Sawtell, N. M.** 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. J. Virol. **71:**5423–5431.
27. **Sawtell, N. M., D. K. Poon, C. S. Tansky, and R. L. Thompson.** 1998. The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. J. Virol. **72:**5343–5350.
28. **Sawtell, N. M., and R. L. Thompson.** 1992. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. J. Virol. **66:**2157–2169.
29. **Steiner, I., J. G. Spivack, R. P. Lirette, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8:**505–511.
30. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
31. **Thompson, R. L., and N. M. Sawtell.** 1997. The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J. Virol. **71:**5432–5440.
32. **Trousdale, M. D., I. Steiner, J. G. Spivack, S. L. Deshmane, S. M. Brown, A. R. MacLean, J. H. Subak-Sharpe, and N. W. Fraser.** 1991. In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. J. Virol. **65:**6989–6993.
33. **Wagner, E. K., G. Devi-Rao, L. T. Feldman, A. T. Dobson, Y. F. Zhang, W. M. Flanagan, and J. G. Stevens.** 1988. Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. **62:** 1194–1202.
34. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. M. Slanina, and H. Ghiasi.** 1988. Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-related RNAs containing open reading frames. J. Virol. **62:**4051–4058.