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The Locus Encompassing the Latency-Associated Transcript of Herpes Simplex Virus Type 1 Interferes with and Delays Interferon Expression in Productively Infected Neuroblastoma Cells and Trigeminal Ganglia of Acutely Infected Mice

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The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) is the only abundant viral transcript expressed in latently infected neurons. LAT inhibits apoptosis, suggesting that it regulates latency by promoting the survival of infected neurons. The LAT locus also contains a newly described gene (AL), which is antisense to LAT and partially overlaps LAT encoding sequences. When human (SK-N-SH) or mouse (neuro-2A) neuroblastoma cells were infected with a virus that does not express LAT or AL gene products (dLAT2903), beta interferon (IFN-β) and IFN-α RNA expression was detected earlier relative to the same cells infected with HSV-1 strains that express LAT and AL. Infection of neuro-2A cells with dLAT2903 also led to higher levels of IFN-β promoter activity than in cells infected with wild-type (wt) HSV-1. In contrast, IFN RNA expression was the same when human lung fibroblasts were infected with dLAT2903 or wt HSV-1. When BALB/c mice were infected with dLAT2903, IFN-α and IFN-β RNA expression was readily detected in trigeminal ganglia (TG) 4 days after infection. These transcripts were not detected in TG of mice infected with wt HSV-1 or dLAT2903R (marker-rescued dLAT2903) until 6 days postinfection. When TG single-cell suspensions from infected BALB/c mice were prepared and incubated in vitro with wt HSV-1 as a source of antigen, TG cultures prepared from mice infected with dLAT2903 produced and secreted higher levels of IFN protein than wt HSV-1 or dLAT2903R. Collectively, these studies suggest that the LAT locus interferes with and delays IFN expression.

Approximately 90% of adults in the United States are infected with herpes simplex virus type 1 (HSV-1) (38, 72). Recurrent ocular HSV-1 is the leading cause of infectious corneal blindness in industrialized nations (40). HSV-1-induced encephalitis (HSE) is a severe form of focal necrotizing encephalitis that affects at least 2,000 individuals each year in the United States (12, 29, 71, 72). Without antiviral therapy, the mortality rate is as high as 70%; but even with antiviral therapy, 20% of these patients die (59, 60). Although HSE was considered a rare disorder, it is now clear that chemotherapy and perhaps other forms of immunosuppression can lead to HSE and/or bilateral acute retinal necrosis (41). Acute infection is typically initiated in the mucocutaneous epithelium, and then HSV-1 establishes latency in sensory neurons located in trigeminal ganglia (TG) or sacral dorsal root ganglia (20, 68). Despite a vigorous immune response during acute infection, latency is established and periodically reactivates.

A single region within the viral long repeats is abundantly transcribed in latently infected neurons, and this transcript is termed the latency-associated transcript (LAT) (6-8, 24, 35, 54, 61, 69, 70). Mice, rabbits, and humans latently infected with HSV-1 express LAT. The primary LAT transcript is approximately 8.3 kb (8, 54, 75). Splicing of the 8.3-kb transcript yields a stable 2-kb LAT and an unstable 6.3-kb LAT. The 2-kb LAT can also be further spliced in infected neurons to yield a 1.4- and a 1.5-kb transcript (30). The majority of the 2-kb LAT is not capped, is not polyadenylated, and appears to be a circularized stable intron (9, 25). Numerous mutants that do not express detectable levels of LAT have been constructed (20, 68). The vast majority of studies using these mutants have demonstrated that LAT significantly enhances the latency reactivation cycle in small animal models (reviewed in references 20 and 21). The HSV-1 McKrae strain is frequently shed in tears of infected rabbits because of spontaneous reactivation (47-51). In contrast, spontaneous reactivation is severely impaired if the LAT gene is deleted (dLAT2903). dLAT2903 contains a deletion from −161 to +1667 relative to the start of the primary 8.3-kb LAT and thus does not express detectable levels of LAT (48, 49). Unlike the wild-type (wt) strain of McKrae, dLAT2903 does not induce high levels of spontaneous reactivation in rabbits (48, 49) or high levels of induced reactivation in mice (46). The spontaneous reactivation phenotype of dLAT2903 in rabbits and the explant-induced reactivation phenotype of dLAT2903 in mice are restored to wild-type levels when the first 1.5 kb of LAT (LAT nucleotides 1 to 1499) driven by the LAT promoter is inserted into an ectopic location in the virus. This indicates that the first 1.5 kb of LAT,

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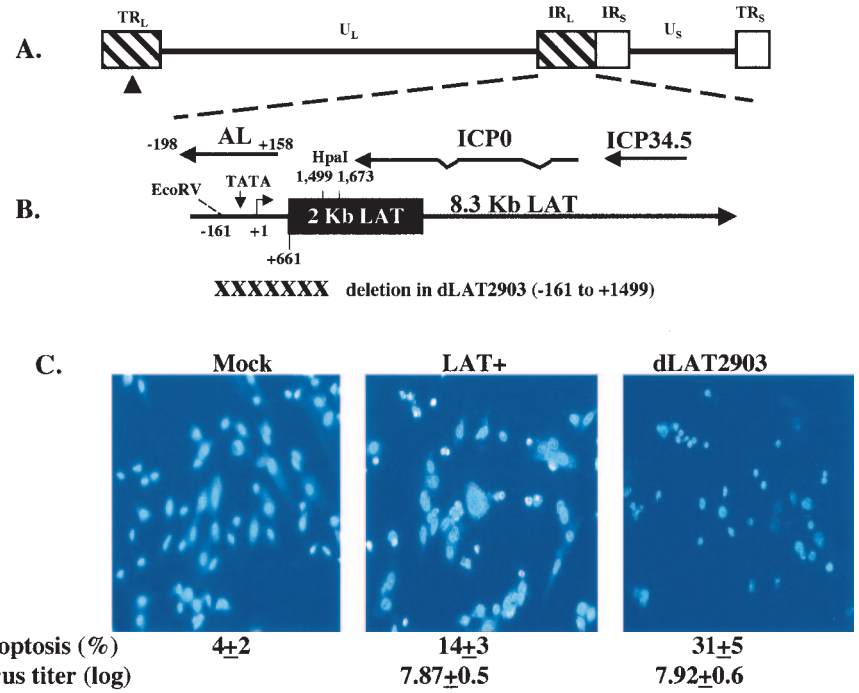

FIG. 1. Morphology of SK-N-SH cells infected with HSV-1. (A) TR_L and IR_L indicate the terminal and inverted long repeats in the HSV-1 genome. IR_S and TR_S indicate the inverted and terminal short repeats. U_L and U_S indicate the unique long and unique short regions. The IR_L is expanded. The TR_L contains the same genes except they are flipped left to right (indicated by the closed triangle). (B) Schematic of genes within the long repeats. The large arrow indicates the primary LAT transcript. The solid rectangle represents the very stable 2-kb LAT intron. The LAT TATA box is indicated by TATA. The start of LAT transcription is indicated by the arrow at +1 (genomic nucleotide 118801). The relative position of the AL transcript is also presented, and the positions of the AL transcript are given using the numbering system of LAT. Several restriction enzyme sites and the relative locations of the ICP0 and ICP34.5 transcripts are shown for reference. The position of the deletion in dLAT2903 (−161 to +1499) is denoted by the Xs. This deletion is present in both copies of the repeats (indicated by the black triangle in the TR_L); thus, dLAT2903 is a LAT null mutant because the core promoter and LAT coding sequences are deleted (46, 47). In addition, dLAT2903 does not express AL gene products (42). (C) SK-N-SH cultures were infected with dLAT2903 or wt HSV-1 (multiplicity of infection [MOI] = 8). One hour after inoculation with the virus at 37°C, the inoculum was removed and the cells were washed three times (10 ml each wash) with phosphate-buffered saline to remove residual virus. Fresh medium was then added to the cultures. At 24 h after infection, Hoechst 33412 staining was performed. Apoptotic cells were counted, and the percentage of apoptotic cells is given. At least 200 cells from each culture were examined from three different experiments. Means were compared by the Tukey-Kramer test at a 5% significance level. The differences between dLAT2903 and LAT^+ (wt or dLAT2903R) or mock infected versus virus infected were significant. At 48 h after infection, the amount of virus was determined by freeze-thawing the infected cells and pelleting the debris. The amount of virus on rabbit skin cells was calculated. The results are the mean of three independent studies.

which contains only the first 837 nucleotides of the stable 2-kb LAT and which does not have the high stability of the 2-kb LAT, is sufficient to support high wild-type levels of reactivation in the rabbit and mouse (19, 49). HSV-1 17syn+ strain mutants with deletions in the LAT promoter and 5′ region of LAT (approximately 1,200 bp) also do not reactivate efficiently in the rabbit eye model (15, 65). These LAT mutants grow with efficiencies similar to those of wt strains in cultured cells and in acutely infected rabbits. LAT may also enhance establishment of latency in mice (56, 64) and rabbits (52), which would increase the pool of latently infected neurons, and consequently may increase the frequency of reactivation from latency. Within the LAT locus, a gene that is antisense to LAT and partially overlaps the 5′ terminus of LAT was recently described and named antisense to LAT (AL) (44). Thus, the LAT locus has at least three distinct genetic elements: the stable 2-kb transcript, functions expressed from the first 1.5-kb LAT coding sequences, and the AL gene.

LAT interferes with apoptosis in transiently transfected cells (1, 16, 42, 45). The ability of LAT to interfere with apoptosis correlates with its ability to promote spontaneous reactivation (16, 19), suggesting the antiapoptotic activity of LAT has biological significance. Recent studies have demonstrated that LAT-expressing plasmids inhibit caspase 8- and caspase 9-induced apoptosis (14, 19). In the context of the viral genome, LAT promotes neuronal survival in TG of infected rabbits (45) and in acutely infected mice (1). Furthermore, LAT reduces apoptosis in infected tissue culture cells (18).

Infection of cultured human cells with HSV-1 leads to production and secretion of alpha interferon (IFN-α) and IFN-β (reviewed in reference 22). HSV-1 glycoprotein D induces IFN-α production in mononuclear cells, resulting in activation of IFN regulatory factor 3 (IRF-3). ICP0, ICP34.5, and Us11 are the known viral genes that block the effects of IFN activation and allow efficient viral replication. A recent study has demonstrated that an ICP0 mutant virus still inhibits IFN-β production, suggesting that an unknown viral function inhibits IFN-β production (34). In addition to possessing potent antiviral activity, IFNs regulate cell growth, apoptosis, inflammation, and the stress response and have immunomodulatory

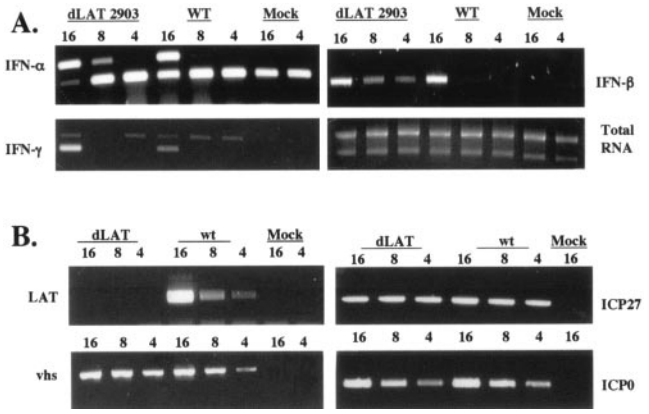

FIG. 2. Gene expression following infection of SK-N-SH cells with HSV-1. SK-N-SH cultures were infected with an LAT mutant (dLAT2903) or a LAT-expressing strain of HSV-1 (wt McKrae or dLAT2903R) at an MOI of 8. Shown in this study are cells infected with wt HSV-1. The results for dLAT2903R and wt McKrae were the same. Total RNA was prepared at the indicated time (hours) after infection using procedures described in Materials and Methods. Two micrograms of total RNA was used for cDNA synthesis using random primers. PCR was then performed using 1/10 of the cDNA reaction mixture and the primers described in Table 2. (A) Analysis of IFN expression in SK-N-SH cells after infection. All IFN-α subtypes were amplified with primers corresponding to the conserved regions of the respective human IFN-α subtypes. The IFN-amplified products were excised and sequenced to confirm they were in fact IFN-amplified bands. The panel marked Total RNA contains 1 μg of total RNA electrophoresed on a 1% formaldehyde gel. The number of PCR cycles used to amplify cDNA for IFN-α was 36 and the number of cycles used to amplify IFN-β or IFN-γ was 34. (B) Analysis of viral gene expression after infection. A total of 31 PCR cycles were used to amplify the respective viral genes. The primers used for PCR amplification are described in Table 1. These results are representative of three independent experiments. vhs, virion host shutoff.

activity (22). There are two main types of IFN, formerly termed type I and type II. Type I IFN includes IFN-α, IFN-β, IFN-ω, and IFN-τ; type II IFN is IFN-γ (22). Most cell types can produce IFN-α and IFN-β, whereas IFN-γ is predominantly produced in natural killer cells, $CD4^+$ T helper cells, and $CD8^+$ cytotoxic T cells. Although there is only one IFN-β gene and one IFN-γ gene, there are numerous IFN-α genes, each encoding slightly different protein isoforms (22). Collectively, the various forms of IFN are important regulators of the innate and adaptive immune response.

In this study, we tested whether the LAT locus influences IFN RNA expression in human neuroblastoma cells (SK-N-SH), mouse neuroblastoma cells (neuro-2A), human lung cells (HEL), and TG of acutely infected BALB/c mice. Infection of SK-N-SH or neuro-2A cells, but not HEL cells, with wt virus delayed IFN-α and IFN-β RNA expression compared to a virus lacking LAT and AL (dLAT2903). When neuro-2A cells were infected with dLAT2903, higher levels of IFN-β promoter activity were expressed relative to cells infected with a wt strain. IFN-β and IFN-α expression in TG of acutely infected mice was also detected earlier when mice were infected with dLAT2903. Finally, there was a decrease in IFN-γ production and secretion by wt HSV-1 infected TG cells, relative to dLAT2903-infected TG cells. These results suggested that LAT or AL gene products delay IFN expression in a neuronal cell-type-specific manner.

MATERIALS AND METHODS

Cells and viruses. Cells were plated at a density of 5×10^5 cells/100-mm plastic dish in Earl's modified Eagle's medium supplemented with 5% fetal bovine serum. All medium contained penicillin (10 U/ml) and streptomycin (100 μg/ml). SK-N-SH (human neuroblastoma cells), neuro-2A (mouse neuroblastoma cells), and human embryonic lung cells (HEL cells) were obtained from the American Type Culture Collection (Rockville, MD) and split in a 1:4 ratio every 3 to 5 days.

All parental and mutant viruses were plaque purified three times and passed only one or two times prior to use. wt McKrae, dLAT2903, and dLAT2903R were described previously (48, 49). Rabbit skin cells were used for preparation of virus stocks. Virus from infected cell lysate was prepared by freeze-thawing three times (−70 to 37°C). Cell debris was removed by centrifugation (10,000 × *g*, 1 h), and the supernatant containing virus was aliqouted and stored at −70°C. Mock-infected cells were prepared in the same fashion. The human IFN-β promoter was not stimulated when undiluted medium from mock-infected cells was added to neuro-2A cells. If virus was pelleted from stock preparations, the supernatant did not activate the IFN-β promoter when added to neuro-2A cells (data not shown).

RNA preparation, reverse transcription-PCR (RT-PCR), and cDNA cloning. TG samples were collected and snap frozen on dry ice. One milliliter of TRIZOL reagent was added (Invitrogen, Carlsbad, CA), and RNA was prepared according to the manufacturer's instructions. For each time point, RNA was prepared from three mice (six total TG). Total RNA was prepared from cultured cells essentially as described by the manufacturer using TRIZOL. RNA samples were subjected to DNaseI digestion to remove trace amounts of contaminating DNA. RNA concentrations were determined initially by measuring the optical density at 260 nm, and the integrity of the total RNA was monitored by running a 1.2% formaldehyde agarose gel with 0.5 to 1 μg of RNA.

First-strand cDNA synthesis was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using equal amounts of total RNA (1 to 2 μg) and oligo$(dT)_{12\text{-}16}$ (for cellular genes) or random hexamers (for HSV-1 genes).

PCR was carried out in a 20-μl reaction mixture that contained 0.5 μM of the designated gene specific primers (Table 2), 1/10 of the cDNA reaction mixture, 2.5 to 5 mM $MgCl_2$, 25 to 100 μM deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase. LAT cDNA was amplified using the GC-rich PCR system (Roche).

IFN-α cDNA was amplified using AccuPrime. The *Taq* DNA polymerase system (Invitrogen) was used for DNA sequencing. PCRs were started with a 3-min incubation at 94°C; 20 to 36 cycles of 30 s at 95°C, 45 s at the annealing temperature, and 72°C for 20 to 45 s; and then 72°C for 7 min. After amplification, 10 μl of each reaction mixture was analyzed by 1.5% agarose gel electrophoresis. The IFN-α band was excised and cloned into the TA Vector system (TOPO TA cloning kit; Invitrogen). Plasmid DNA was purified using a Plasmid Mini Kit (QIAGEN) and then sequenced. Sequence data were analyzed using GenBank (National Center for Biotechnology Information).

Plasmid constructs. The human IFN-β promoter (−110 to + 20) linked to the bacterial chloramphenicol acetyltransferase (CAT) gene was obtained from Stavros Lomvardas (Columbia University, New York). This minimal promoter contains the essential promoter elements that are activated by virus infection or other IFN signaling pathways (36). The pISRE plasmid is a CAT reporter construct that contains four consensus interferon-stimulated response elements (ISRE) from the ISG15 promoter that is upstream of a minimal HIV promoter construct. The pISRE plasmid was obtained from L. Zhang (University of Nebraska). CAT activity was examined as described previously (16, 17, 74).

Mouse studies. Adult female BALB/c mice (54 to 82 days old) were obtained from Charles River Laboratory and were used for these studies. Mice were ocularly infected with an infectious dose of 2×10^5 PFU/eye. Mice were bilaterally infected without scarification by placing the virus (2 μl of inoculum) into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s. Prior to infection, mice were lightly anesthetized with isoflurane (Alocarbon Laboratories, River Edge, N.J.). The mice were observed daily during the studies and were euthanized by CO_2 inhalation at the indicated time points.

For IFN protein detection, sex- and age-matched BALB/c mice were either mock infected (mock) or infected with dLAT2903, wt HSV-1, or dLAT2903R. Five days postinfection, all animals were sacrificed, and TG were aseptically collected and treated with collagenase IV (Sigma). Single-cell suspensions were prepared and washed three times in sterile Dulbecco's modified Eagle's com-

A.

IFN-α	McKrae Clones (31)	McKrae (%)	dLAT2903 Clones (28)	dLAT2903 (%)
α-1	3	9.6	1	3.5
α-5	10	32.2	15	53.3
α-6	0	0	1	3.5
α-7	2	6.4	1	3.5
α-10	5	16	5	17.8
α-14	3	9.6	0	0
α-16	1	3.2	1	3.5
α-17	3	9.6	1	3.5
α-21	4	12.9	3	10.7

B.

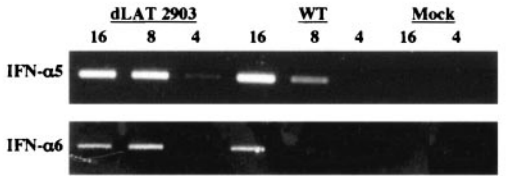

FIG. 3. Induction of different IFN-α subtypes in SK-N-SH cells infected with McKrae or dLAT2903. (A) The IFN-α cDNAs generated by RT-PCR from SK-N-SH cells infected with McKrae or dLAT2903 (Fig. 2A) were cloned as described in Materials and Methods. Randomly selected clones were sequenced and the sequence analyzed to identify which IFN-α subtype was present. (B) Primers that specifically amplify IFN-α5 or IFN-α6 were synthesized (Table 2) and used for RT-PCR as described in Materials and Methods.

plete medium. The TG cell suspensions were placed in triplicate wells at 10^6 total cells per well and either left untreated (mock or negative control) or stimulated in vitro at 37°C for 96 h using heat-inactivated HSV-1 as the antigen. As a positive control, TG cell suspensions were stimulated with concanavalin A (data not shown) (Sigma). For each condition, the IFN protein produced and secreted in the supernatant of cultured cells was quantified by an enzyme-linked immunosorbent assay (ELISA) sandwich kit (Pharmingen, San Diego, CA).

RESULTS

LAT influences cytotoxicity and apoptosis in SK-N-SH cells. SK-N-SH cells are human neuroblastoma cells that have been used to examine apoptosis after HSV-1 infection (10, 11). In this study, we compared the level of apoptosis induced in SK-N-SH cells by wt HSV-1 (McKrae strain), a LAT deletion mutant (dLAT2903), or the marker-rescued dLAT2903 (dLAT2903R) (Fig. 1A and B). dLAT2903 contains a deletion from −161 to +1667 relative to the start of the primary 8.3-kb LAT and thus does not express detectable levels of LAT (48, 49) or the AL transcript (44). SK-N-SH cells productively infected with dLAT2903 has consistently led to >30% apoptotic cells at 24 h after infection, as judged by Hoechst 33342 staining (Fig. 1C). In contrast, SK-N-SH cells infected with wt McKrae or dLAT2903R (the rescued LAT mutant that behaves as wt McKrae) had approximately 14% apoptotic cells at 24 h postinfection. The differences in apoptosis between the LAT^+ (wt McKrae or dLAT2903R)-infected cells were significantly different ($P < 0.05$) from levels of apoptosis in cells infected with dLAT2903 when these differences were analyzed using a Tukey-Kramer test at a 5% significance level. Many of the cells infected with dLAT2903 contained nuclei that appeared to have "collapsed" by 24 h postinfection. Although LAT played a role in inhibiting apoptosis in productively infected SK-N-SH cells, the end point virus titers were similar regardless of whether cells were infected with wt McKrae, dLAT2903R, or dLAT2903. The fact that HSV-1 contains several antiapoptosis genes (3) and that differences between dLAT2903 versus wt HSV-1 were not detected until 24 h after infection may explain the similarity in virus titers.

LAT delays IFN-α and IFN-β RNA expression in SK-N-SH cells. When SK-N-SH cells were infected with dLAT2903, IFN-β RNA was detected at 4, 8, and 16 h after infection (Fig. 2A; right). In contrast, IFN-β RNA expression was not readily detected until 16 h after infection when SK-N-SH cells were infected with wt McKrae (Fig. 2A) or dLAT2903R (data not shown). In addition, SK-N-SH cells infected with dLAT2903 contained readily detectable levels of IFN-α RNA by 8 h after infection. Although a small amount of IFN-α RNA expression was detected at 8 h after infection with LAT^+ viruses (i.e., either wt McKrae or dLAT2903R) (Fig. 2A; left), it was not easily detected until 16 h after infection. IFN-γ was not detected until 16 h after infection, regardless of whether SK-N-SH cultures were infected with dLAT2903 or wt HSV-1. The intensity of the IFN-γ-amplified cDNA was consistently higher in dLAT2903-infected SK-N-SH cells. As expected, IFN-α, IFN-β, and IFN-γ RNA was not detected in mock-infected cells.

For these studies, we used readings at an optical density of 260 nm and total RNA as methods to ensure that the same amounts of total RNA were used for RT-PCR (Fig. 2A). As another control, we examined the levels of four viral transcripts (virion host shutoff, ICP0, ICP27, and LAT) in productively

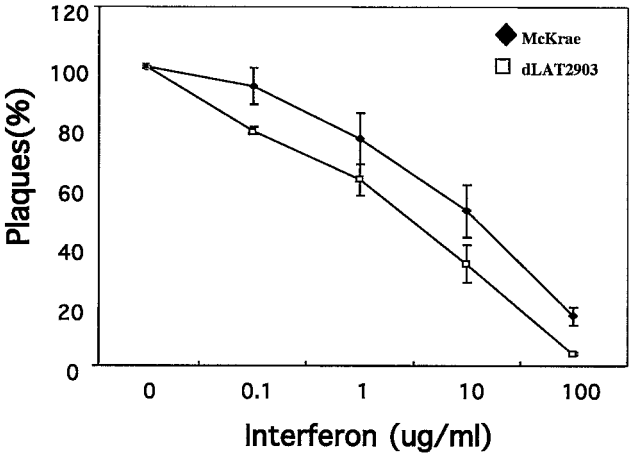

FIG. 4. Pretreatment of SK-N-SH cells with IFN inhibits HSV-1 plaque formation. SK-N-SH cells were seeded in six-well plates, and the cells reached 80 to 90% confluence. On the next day, SK-N-SH cultures were treated with IFN-β (0.1 to 1,000 U) (Calbiochem 407297) for 6 h and then infected with HSV-1 (MOI = 0.01). One hour later, the medium was replaced with Earl's modified Eagle's medium containing 10% fetal calf serum and the same concentration of IFN used as in the 6-h pretreatment. The number of plaques was counted 48 to 72 h after infection. The number of plaques without IFN-β was set at 100%. Although IFN-β treatment had a slight effect on the size of plaques, we did not observe dramatic differences between dLAT2903 and LAT$^+$ strains (data not shown). These results are the average of five different experiments.

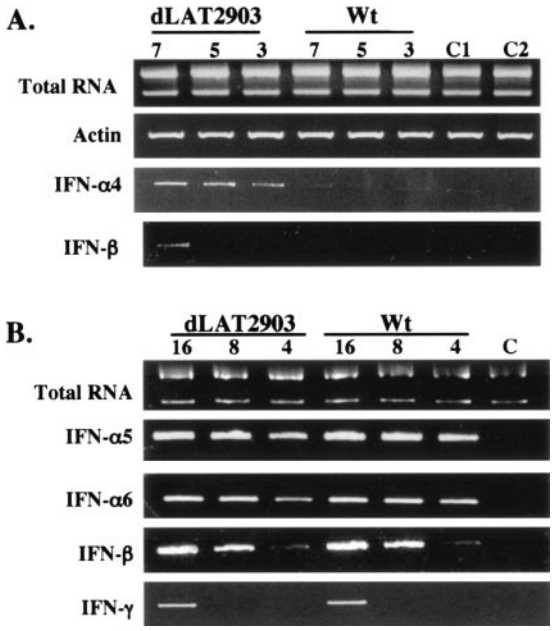

FIG. 5. Induction of IFN-α in HEL or neuro-2A cells infected with McKrae or dLAT2903. Neuro-2A cells (A) or HEL cells (B) were infected with dLAT2903 or a LAT-expressing strain of HSV-1 (wt McKrae or dLAT2903R) at an MOI of 8. Total RNA was prepared at the indicated time (hours) after infection. Two micrograms of total RNA was used for cDNA synthesis using random primers. PCR was then performed using 1/10 of the cDNA reaction mixture and the primers described in Table 2.

infected SK-N-SH cultures. Cultures infected with dLAT2903 or wt McKrae exhibited no dramatic qualitative or quantitative difference in ICP0, ICP27, or virion host shutoff RNA expression (Fig. 2B). This result was consistent with the finding that similar virus titers were produced in cells infected with dLAT2903 or wt HSV-1 (Fig. 1). LAT was not expressed when cultures were infected with dLAT2903 because the core LAT promoter and the first 1.5 kb of LAT coding sequences were deleted (48). As expected, LAT was detected as early as 4 h after infection following infection with wt McKrae. In summary, these studies indicated that sequences within the LAT locus played a role in delaying expression of IFN-α RNA and IFN-β RNA in SK-N-SH cells.

Identification of IFN-α subtypes in SK-N-SH cells after infection. The IFN-α gene family has extensive similarity at the nucleotide level and contains numerous family members that encode slightly different protein subtypes (22). IFN-α subtypes are expressed in a cell-type-dependent fashion, suggesting they have different effects on certain cells (13). In contrast, there is a single IFN-β gene and a single IFN-γ gene. The primers that were used to amplify human IFN-α RNA will amplify all IFN-α subtypes (33). Thus, it was possible to compare the distribution of IFN-α subtypes in SK-N-SH cells after infection with dLAT2903 or wt McKrae by cloning and sequencing the PCR-amplified IFN-α cDNA band. When cultures of SK-N-SH cells were infected with dLAT2903, approximately 53% of the IFN-α subtypes expressed were IFN-α5, whereas 32% of the clones were IFN-α5 when cells were infected with wt McKrae (Fig. 3A). Subtle differences were also detected among other isotypes, for example, IFN-α1, IFN-α6, and IFN-α14.

To further test whether sequences within the LAT locus delayed expression of IFN-α subtypes, primers that specifically amplify IFN-α5 or IFN-α6 were designed and tested. The IFN-α5-specific primers amplified a cDNA product at 4 h after infection when SK-N-SH cells were infected with dLAT2903 but not until 8 h after infection with wt McKrae (Fig. 3B). IFN-α6-amplified cDNA products were readily detected as early at 8 h after infection with dLAT2903 but not until 16 h after infection with wt McKrae.

Pretreatment of SK-N-SH cells with IFN-β had a dramatic impact on the number of plaques formed in SK-N-SH cells (Fig. 4), confirming that IFN has antiviral activity against HSV-1 in these cells. The effects of IFN-β on virus replication were slightly higher when cultures were infected with dLAT2903 rather than wt McKrae. Although the LAT locus delayed IFN RNA expression in productively infected SK-N-SH cultures, pretreatment of SK-N-SH cells with IFN did not show a dramatic difference between the growth properties of dLAT2903 and LAT$^+$ strains.

Analysis of IFN expression in neuro-2A and HEL cells. To test whether the LAT locus influenced IFN expression in other cell types, we infected mouse neuroblastoma cells (neuro-2A) or human lung cells (HEL) with dLAT2903 or wt McKrae and examined IFN RNA at different times after infection. In keeping with the results obtained with SK-N-SH cells, qualitative differences in IFN-β and IFN-α4 RNA expression were observed in neuro-2A cells (Fig. 5A). In contrast to the results obtained with SK-N-SH or neuro-2A cells, no obvious differences in IFN-β RNA expression were detected in HEL cells infected with dLAT2903 versus wt McKrae (Fig. 5B). Further-

TABLE 1. Activation of the human IFN-β promoter and ISRE-dependent transcription, following infection with HSV-1[a]

Promoter	dLAT2903				dLAT2903R			
	1	2	3	4	1	2	3	4
IFN-β	3.4	5.5	4.1	4.3	1.5	2.9	1.7	1.5
pISRE	6.5	3.9	5.7		6.8	3.4	5.6	

[a] Cultures of neuro-2A cells were transfected with the designated promoter constructs (1 μg) plasmid DNA. At 24 h after transfection, the cultures were infected with the designated viruses using an MOI of 4. At 40 h after transfection, the total cell lysate was collected and CAT activity was measured. The levels of acetylated chloramphenicol was measured using a BioRad Molecular Imager FX. The levels of promoter activity in mock-infected cells were set and the fold of stimulation was then calculated for each promoter. The results of hree independent experiments are shown for the two promoters. Four experiments (1 to 4) are shown for each construct.

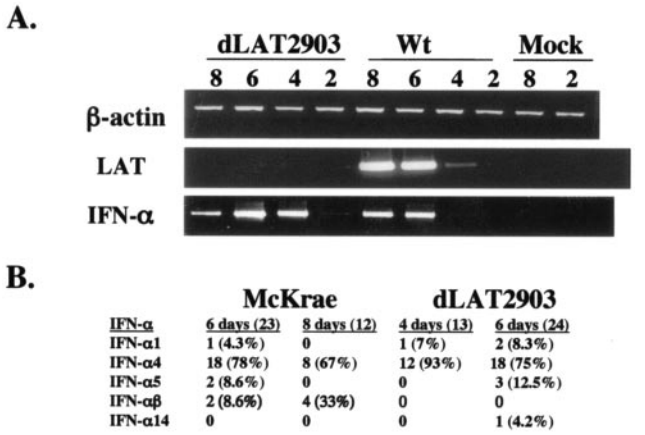

FIG. 6. Induction of IFN-α in TG of mice infected with McKrae or dLAT2903. (A) BALB/c mice were infected with HSV-1 as described in Materials and Methods. At the indicated times after infection, TG were harvested and total RNA prepared (TG from 2 mice/time point). RT-PCR using the designated primers was performed (Table 2). All mouse IFN-α subtypes were amplified using consensus primers that are located within the conserved region of the IFN-α RNA. The top panel shows the corresponding levels of β-actin mRNA as a loading control. (B) The IFN-α cDNA band that contains a mixture of the IFN-α subtypes was excised and cloned, and the insert was sequenced. The band at 4 days after infection (McKrae) did not yield clones, which was expected because we were unable to amplify IFN-α cDNAs at this time.

more, no dramatic differences were observed using primers that amplified IFN-α5 or IFN-α6 (Fig. 5B) or primers that amplified all IFN-α cDNAs (data not shown). As with SK-N-SH cells, IFN-γ was not detected in HEL cells until 16 h after infection with either dLAT2903 or wt HSV-1. Viral gene expression and virus titers were nearly identical in HEL and neuro-2A cells infected with dLAT2903 or wt McKrae (data not shown). These findings suggested that cell-type-specific factors played a role with respect to whether the LAT locus delayed IFN RNA expression.

Additional studies were performed to test whether infection of cultured cells with dLAT2903 contained higher levels of IFN-β promoter activity than cells infected with dLAT2903R. For these studies, two promoter constructs were used: the human IFN-β promoter and a minimal HIV promoter that contains four copies of a consensus ISRE. Neuro-2A cells were used for these studies because they can be readily transfected, whereas HEL and SK-N-SH cells do not transfect efficiently. In four separate experiments, IFN-β promoter activity was higher in neuro-2A cells infected with dLAT2903 versus dLAT2903R (Table 1). In contrast, pISRE promoter activity was not different regardless of whether the cells were infected with dLAT2903 or dLAT2903R. The data was analyzed using a Student's *t* test (two tailed). The IFN-β promoter activity in cells infected with dLAT2903 versus dLAT2903R was significantly different ($P = 0.0046$), but it was not when comparing pISRE promoter activity ($P = 0.94$). Although these studies support the findings that a factor encoded by the LAT locus inhibits the IFN response, they also suggest that not all IFN signaling pathways are inhibited by the LAT locus.

The LAT locus delayed IFN expression in TG of infected mice. Although the data obtained from neuroblastoma cells suggested that the LAT locus delayed IFN RNA expression during productive infection (Fig. 2 to 5A), it was also clear that the LAT locus had no dramatic effect on IFN RNA expression in HEL cells (Fig. 5B). Since LAT has an effect on the latency reactivation cycle but does not have a dramatic effect on productive infection (20, 21), we hypothesized that if the LAT locus played a role in delaying IFN RNA expression, this might only occur in neuronal cell types. To further test whether LAT or other genes within the LAT locus influenced IFN RNA expression in TG of acutely infected mice, adult female BALB/c mice were infected with LAT^+ strains or dLAT2903, and expression of IFN RNA in TG was analyzed by RT-PCR. We initially examined IFN-α expression in TG of infected mice (Fig. 6). Mouse-specific primers were used for this study because there are differences in mouse and human sequences (Table 2). IFN-α RNA expression was readily detected at 4 days after infection when mice were infected with dLAT2903 (Fig. 6A). In contrast, IFN-α RNA expression was not detected until 6 days after infection with wt McKrae (Fig. 6A) or dLAT2903R (data not shown). As expected, LAT was not detected in mice infected with dLAT2903 but was readily detected in mice infected with wt HSV-1. In keeping with previous studies (46), dLAT2903 and wt McKrae grew to similar titers in the eyes and TG of infected mice (data not shown).

The mouse-specific IFN-α primers were designed to amplify all IFN-α subtypes, allowing us to compare the distribution of subtypes expressed in TG after infection. The IFN-α RT-PCR band was eluted from the agarose gel and cloned, and the plasmid inserts were sequenced. At 4 days after infection, no clones were obtained when the region of the gel encompassing the amplified IFN-α band was excised from samples prepared from mice infected with wt McKrae (Fig. 6B). Twelve of 13 clones (>92%) that were obtained from the RT-PCR products in mice infected with dLAT2903 at 4 days after infection and 18 of 24 (75%) at day 6 after infection were IFN-α4. Similarly, 78 and 67% of the clones from mice infected with wt McKrae were also IFN-α4 on days 6 and 8 after infection, respectively. Interestingly, mice infected with wt McKrae contained 8.6 or 33% IFN-αB subtypes at 6 or 8 days after infection, respectively, whereas IFN-αB was not detected in mice infected with dLAT2903 (Fig. 6B).

Mice infected with dLAT2903 expressed IFN-β in TG as early as 4 days after infection, whereas mice infected with wt McKrae or dLAT2903R did not express IFN-β until 6 days

TABLE 2. Nucleotide sequences of PCR primers

Gene	Primer sequences	Product (bp)	Accession no.
HSV-1			
VHS	TGCTACATTCCCACGATCAA, AGGTCCTCGTCGTCTTCGTA	346	AF007815
ICP0	ACAGACCCCCAACACCTACA, GCGTATGAGTCAGTGGGGA	150	AF431736
ICP27	CCCTTTCTCCAGTGCTACCTGAA, GTGCGTGTCTAGGATTTCGATC	256	
LAT	TTGGCGGTAACCCCGATTGTTTATCTCAGG & TCGTTCCGTCGCCGGGATGTTTCGTTCGT	200	
Human			
IFN-α	AGAATCTCTCCTTTCTCCTG, TCTGACAACCTCCCAGGCACA	369	
IFN-α6	CTGGACTGTGATCTGCCTCA, CTTCAGCCTTCTGGAACTGG	169	gi:11128014
IFN-α5	CCAGTTCCAGAAGG CTCAAG, TGTCTTCCACTCCAACCTCC	197	gi:4504596
IFN-β	TGGGAGGATTCTGCATTACC, CAGCATCTGCTGGTTGAAGA	200	gi:4504602
IFN-γ	TGACCAGAGCATCCAAAAGA, ATATTGCAGGCAGGACAACC	280	gi:10835170
Mouse			
IFN-α	TCTCCTGCCTGAAGGACAGG, GAGCAGAAGTCTGGA	320	
IFN-αB	TGGCAGTGATGAGCTACTGG, ATCTGCTGGGTCAGCTCAGT	240	gi:6680370
IFN-α14	TGCTGGTGATGAGCTACTGG, GAGCCTTCTTGATCTGCTGG	200	gi:29468971
IFN-α4	CTGGTCAGCCTGTTCTCTAGGATGT, TCAGAGGAGGTTCCTGCATCAC	314	
IFN-β	CCCTATGGAGATGA CGGAGA, TCCCACGTCAATCTTTCCTC	222	gi:6754303
IFN-γ	GAAAAGGAG TCGCTGCTGAT, AGATACAACCCCGCAATCAC	319	gi:2850152
p53	GTACCTTATGAGCCACCCGA, CTGTAGCATGGGCATCCTTT	446	gi:6755880
β-Actin	GTGGGGCGCCCCAGGCACCA, CTCCTTAATGTCACGCACGATTTC	550	

[a] The primers directed against glycoprotein C (gC), β-actin, and LAT were described previously (41). The human IFN-α primer that amplifies all human subtypes of IFN-α was described previously (34). The mouse IFN-α primer that amplifies all human subtypes of IFN-α was designed based on the consensus sequence of the respective IFN-α subtypes in mice. The mouse IFN-α4 primers were described previously (65). All primers are presented in a 5′-to-3′ direction. The first primer is the forward primer and the second is the reverse primer.

after infection (Fig. 7). We also synthesized IFN-α-specific primers that amplified only IFN-α4 (67), IFN-α14, or IFN-αB. As predicted from the distribution of IFN-α subtypes (Fig. 6B), IFN-α4–IFN-α14 products were detected earlier in TG of mice infected with dLAT2903 (4 days after infection) versus mice infected with wt McKrae or dLAT2903R (6 days after infection). Furthermore, IFN-αB was detected in TG of mice infected with wt McKrae or dLAT2903R but not dLAT2903 (Fig. 7), which was consistent with the sequencing analysis of IFN-α subtypes expressed after infection (Fig. 6B). IFN-γ RNA expression was readily detected in TG of mice infected with dLAT2903 at 2 days after infection, whereas it has been difficult to detect IFN-γ RNA in TG of mice infected with dLAT2903R or wt HSV-1 until 4 days after infection. ICP27, β-actin, and p53 RNA expression was similar in TG of infected mice, regardless of whether mice were infected with dLAT2903, dLAT2903R, or wt HSV-1. This result is consistent with the finding that similar levels of virus were detected in TG of mice regardless of whether the mice were infected with LAT^+ virus strains or strains lacking LAT. In summary, this study indicated that the LAT locus encodes a factor that directly or indirectly delayed RNA expression of certain subtypes of IFN-α and IFN-β in TG of infected mice. Furthermore, wt HSV-1, but not dLAT2903, appeared to induce expression of IFN-αB RNA in TG of acutely infected mice.

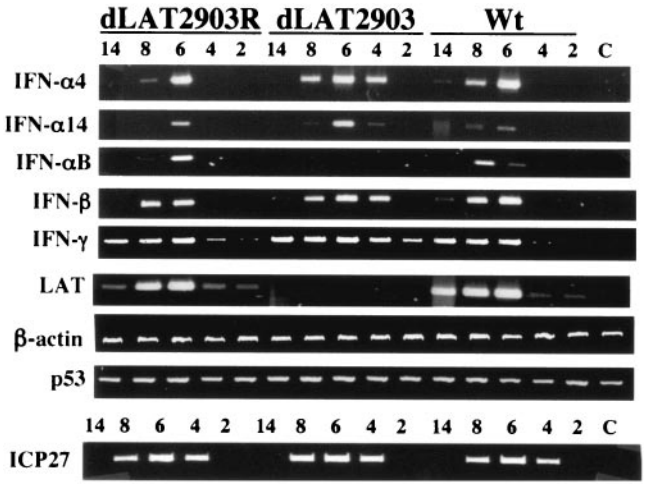

FIG. 7. Expression of IFN in TG of mice infected with dLAT2903 or LAT^+ strains of HSV-1. BALB/c mice were infected with HSV-1 as described in Materials and Methods. At the indicated times after infection, TG were harvested, and total RNA was prepared. RT-PCR using the designated primers (Table 1) was performed. As loading controls, the expression levels of β-actin and p53 are shown in the bottom panels. The results are representative of two different experiments.

Decreased IFN-γ production by wt HSV-1- and dLAT2903R-infected TG cells compared to dLAT2903-infected TG cells. Next, we sought to determine whether the presence of the LAT locus would interfere with the expression of interferon at the protein level. Groups of BALB/c mice were either mock infected or infected with dLAT2903, wt HSV-1, or dLAT2903R. Five days postinfection, TG were removed from each group, single-cell suspensions were made, and 10^6 TG cells/well were either left untreated (mock) or stimulated in vitro with wt HSV-1. The amount of IFN-γ protein produced in the supernatant of stimulated TG cells was then detected by a sandwich ELISA and normalized to the total number of TG cells (i.e., 10^6). As shown in Fig. 8, with or without in vitro restimulation, TG cells from the dLAT2903 group produced significantly

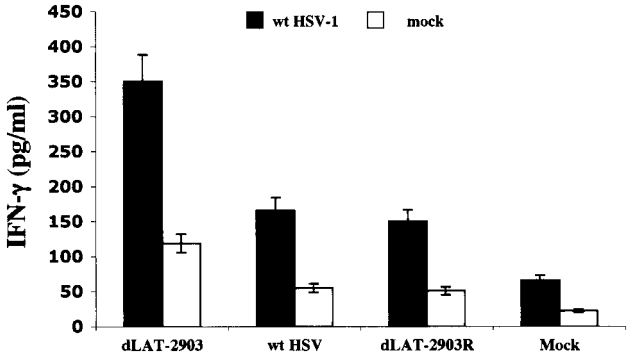

FIG. 8. Decreased IFN secretion in TG from mice infected with wt HSV-1 or dLAT2903R viruses. TG single-cell suspensions were obtained from age- and sex-matched mice infected with dLAT2903, wt HSV-1, or dLAT2903R at 5 days postinfection. Noninfected mice were used as a negative control. For each reaction, an equivalent number of cells from two TG were either left untreated (white bars) or incubated in vitro at 37°C with heat-inactivated wt HSV-1 as a source of antigen (black bars). The secreted IFN was measured 96 h later by a standard sandwich ELISA assay. The results are representative of two different experiments.

more IFN-γ than TG cells from the LAT^+ groups (dLAT2903R or wt) ($P < 0.001$). All three infected groups also produced significantly more IFN-γ than the mock-infected group ($P < 0.001$) Preliminary IFN-γ enzyme-linked immunospot assays suggested a higher frequency of IFN-γ-secreting cells in TG cells lacking LAT than in LAT^+ TG cells (unpublished data). Taken together, these results suggest that the LAT locus directly or indirectly impairs IFN expression at both the RNA and the protein level.

DISCUSSION

In this study, we provide evidence that a factor encoded by the LAT locus delayed RNA and protein expression of certain IFNs in neuroblastoma cells (SK-N-SH or neuro-2A) and TG of infected mice but not in HEL cells. Consistent with these studies, we also demonstrated that the LAT locus reduced IFN-β promoter activity in infected neuro-2A cells. The LAT deletion mutant that was used for this study has at least three genetic elements deleted: (i) the stable 2-kb LAT that has been reported to encode a protein capable of replacing ICP0 functions during reactivation from latency (62, 63); (ii) the first 1.5 kb of LAT that contains only a portion of the stable 2-kb stable LAT, but a fragment that is sufficient for high levels of spontaneous reactivation in the rabbit eye model (49) or TG explant-induced reactivation in the mouse (46); and (iii) a transcript located within the 5′ end of LAT and the LAT promoter that is antisense to LAT (the AL transcript) (44). In addition to the first 1.5-kb LAT promoting spontaneous reactivation (48, 49), this same fragment inhibits caspase 8-induced apoptosis very efficiently (43). A LAT fragment that contains the first 2.8 kb of LAT coding sequences (includes the stable 2-kb LAT) efficiently inhibits caspase 9-induced apoptosis (19) but does not inhibit caspase 8-induced apoptosis any better than the 1.5-kb LAT fragment (48, 49). The function of the AL gene is currently unknown. Studies are in progress to test whether LAT or the AL gene delays IFN RNA expression.

In the context of productive infection in SK-N-SH cells, the ability of the LAT locus to stimulate virus production was marginal when cultures were pretreated with IFN-β (Fig. 4). Since HSV-1 encodes several other genes that inhibit the deleterious effects of IFN (22, 34), these genes apparently compensate for the loss of this factor or this factor is only important during a specific stage of the latency reactivation cycle. Interestingly, a recent report demonstrated that an ICP0 mutant virus retains the ability to inhibit IFN-β production (34), which supports our finding that the LAT locus inhibits IFN-β expression in SK-N-SH cells. If LAT is responsible for delaying IFN RNA expression, we suggest that LAT RNA, not a LAT protein, inhibits IFN RNA expression. There is precedence for non-protein-encoding RNAs that inhibit the IFN response. For example, a small Epstein-Barr virus RNA is expressed in latently infected B cells and has been reported to inhibit the IFN response (39). An adenovirus nonpolyadenylated RNA can also repress the IFN response (57). Finally, a cellular RNA that does not appear to encode a protein inhibits IFN-induced apoptosis in HeLa cells (58).

In contrast to the results obtained in SK-N-SH cells, neuro-2A cells, or TG of acutely infected mice, LAT did not delay IFN RNA expression in human lung fibroblasts (HEL) (Fig. 5B). Mutation of the splice acceptor site or the splice donor site that generates the 2-kb LAT drastically reduces expression of the 2-kb LAT during productive infection but has little effect on expression during latency (2). This study suggests that processing of LAT is cell type specific and may regulate LAT function. It is also possible that AL gene products are expressed at higher levels in neuronal cell types or that the putative AL protein has novel functions in neuronal cell types.

The finding that the LAT locus delayed expression of IFN-α4 RNA and reduced the frequency of IFN-α4 RNA in TG of infected mice is significant because IFN-α4 expression is an "immediate early" response to virus infection (31). After virus infection, phosphorylation of interferon regulatory factor 3 (IRF3) activates IFN-α4 gene expression. IFN-α4 and IFN-β subsequently activate expression of other interferon regulatory factors (IFN-stimulated gene factor 3 and IRF7, for example) that stimulate expression of the remaining IFN-α subtypes. More than a dozen mouse IFN-α genes are clustered on chromosome 4 (37, 53). The presence of multiple IFN-α genes is believed to be important because immediate early expression of IFN-α4 provides a rapid response to virus infection, while sequential induction of delayed IFN-α genes amplifies the protective response to virus infection. In general, it is believed that IFN-α subtype expression in mice is mediated by differences in the respective promoters of the genes. Our findings suggest that the LAT locus regulates expression of specific IFN-inducible genes for the following reasons. (i) The LAT locus repressed IFN-β, but not pISRE, promoter activity in neuro-2A cells. (ii) The LAT locus stimulated IFN-αB RNA expression but inhibited IFN-α4 RNA expression in TG of infected mice.

In addition to blocking protein synthesis and establishing an "antiviral" state, several interferon-stimulated genes have pro-apoptosis activity(reviewed in reference 5). For example, IFN induces RNA expression of TRAIL, Fas, Xaf-1, caspase 4, caspase 8, RIDs, PKR, IRF-1, PML, RNase L, gelectin-9, OAS-1 9-2 isozyme, and death-associated protein kinases. Of

particular note, the caspase 8 promoter is strongly regulated by an interferon-sensitive response element in neuroblastoma cells (4). Regardless of cell type or tissue histology, induction of apoptosis by all IFN subtypes (IFN-α, IFN-β, or IFN-γ) involves caspase 8 signaling, activation of the caspase cascade, release of cytochrome *c*, and DNA fragmentation (5). Production of IFN during infection also regulates cytotoxic T lymphocyte responses (32, 55, 66). A long-term inflammatory response occurs in TG of mice infected with HSV-1 (23, 26–28) or cattle infected with bovine herpesvirus type 1 (73), and interferon and other cytokines are produced. In TG of rabbits infected with dLAT2903, there is an increase in the number of infiltrating cells (45). Furthermore, in mice there was a relative increase in the level of IFN secretion by dLAT2903-infected TG cells (Fig. 8). This suggests that the ability of the LAT locus to decrease IFN RNA and protein levels resulted in reduced lymphocyte infiltration in TG. In addition to the known effect of IFN in promoting a type 1 T-helper-cell (Th1) response, a critical arm in HSV immunity, the interference of LAT with IFN expression may also decrease the probability of immune mediated killing of infected neurons.

We hypothesize that the ability of the LAT locus to delay the IFN response is important because it promotes neuronal survival by inhibiting apoptosis, preventing immune recognition and allowing normal neuronal functions to continue after infection. During the establishment and maintenance of latency, the other virus-encoded IFN regulators would not be expressed. Since LAT and AL are regulated by sequences within the LAT promoter, these functions are expressed at higher levels during latency, in particular, LAT. The above-mentioned effects are not mutually exclusive, and both may counteract the host immune response and support efficient viral replication and reactivation. Thus, inhibiting IFN expression may enhance the long-term survival of infected neurons during the latency reactivation cycle.

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