Effect of the Transcription Start Region of the Herpes Simplex Virus Type 1 Latency-Associated Transcript Promoter on Expression of Productively Infected Neurons In Vivo

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It has been previously reported that the latency-associated transcript (LAT) promoter contains a DNA sequence at the LAT transcription start site which resembles the ICP4 consensus DNA binding site and that this site allows ICP4-mediated downregulation of the LAT promoter in transient assays (A. H. Batchelor and P. O'Hare, *J. Virol.* 64:3269–3279, 1990). We have confirmed these data by showing that an ICP4-expressing plasmid will downregulate *lacZ* expression from a plasmid containing the LAT promoter and transcription start site (pJA1) and does not downregulate *lacZ* expression from a plasmid in which the start site has been mutagenized (pWAG15). To determine the role of the LAT transcription start site in regulating LAT promoter activity in the context of the virus, two recombinant viruses, KOS-1 and KOS-15, were studied. KOS-1 contains an 863-bp portion of the LAT promoter, including the LAT cap site, fused to the *lacZ* gene and inserted into the gC locus (T. P. Margolis, F. Sedarati, A. T. Dobson, L. T. Feldman, and J. G. Stevens, *Virology* 189:150–160, 1992). The second virus (KOS-15) was constructed in identical fashion, using plasmid pWAG-15, which is not downregulated by ICP4. Vero cells productively infected with KOS-15 produce 10-fold more β -galactosidase than do those infected with KOS-1. In murine dorsal root ganglia acutely infected with KOS-1, only 1.2% of dorsal root ganglion neurons that expressed viral antigen also expressed β -galactosidase. In contrast, in KOS-15-infected mice, β -galactosidase was detected in 18% of viral antigen-positive neurons. Similar findings were observed in trigeminal ganglia acutely infected with KOS-1 and KOS-15. Thus, the region encompassing the LAT transcription start site appears to play an important role in repression of the LAT promoter activity not only in vitro but also in acutely infected neurons in vivo. These results suggest that during productive infection with HSV-1, LAT expression is tightly regulated.

Herpes simplex virus (HSV) can infect and remain latent in a variety of peripheral and central nervous system neurons (18, 37, 46, 47). During latency, lytic cycle genes are not expressed and RNA accumulates from a single region of the viral genome (4, 5, 16, 36, 43, 48, 49). The RNA detectable during latency is composed of two or three very stable molecules ranging in size from 1.4 to 2.0 kb, which have been called LAT, for latency-associated transcripts; the remaining RNA can only be detected by in situ hybridization (8, 13, 51–53). In total, these RNAs are derived from a transcription unit which is approximately 8.3 kb in size and are initiated 688 bp upstream of the very stable LAT RNAs (10, 26, 45). Expression of LAT region RNA is not essential for establishment of a latent infection, but it might affect the efficiency with which a latent infection is established (19, 21, 37, 45). Instead, LAT region expression appears to be necessary for efficient reactivation from the latent state (3, 7, 17, 25, 26).

The HSV-1 transactivating protein ICP4 plays an essential role in the viral replication cycle (6). The biological role of ICP4 in infection is to increase the transcription rate of early and late viral genes, as shown by Godowski and Knipe (15). Like simian virus 40 large T antigen, it also contains an autoregulation function in which ICP4 protein is able to bind to its own cap site and repress further ICP4 gene transcription (29–32, 35). An interesting feature of the LAT promoter is the

presence of a near-consensus ICP4 binding site coincident with the LAT transcription start site. Batchelor and O'Hare showed that ICP4 significantly downregulates LAT promoter-driven gene expression in transient transfection assays (1).

Recent studies showed that productively infected neurons appear to negatively regulate LAT promoter-driven gene expression (28, 41, 42). Using recombinant viruses containing LAT promoter-*lacZ* gene fusions such as KOS-1, Margolis et al. were able to show that early in the course of an acute neuronal infection, less than 0.2% of neurons that contained viral antigen also expressed LAT promoter-driven β -galactosidase (28). Studies by Speck and Simmons also show an early divergence of the latent and productive pathways of viral gene transcription (41, 42). Earlier work with viral mutants show a similar separation; e.g., Kosz-Vnenchak et al. (23, 24) showed that thymidine kinase-negative mutants express LAT in sensory neurons without expression of other viral genes, and Valyi-Nagi et al. (50) showed that a VP16 mutant expressed LAT in sensory neurons without expression of other viral genes. This was further illustrated by the lack of viral gene expression in neurons infected with an ICP4 deletion virus (40).

The fact that expression of the *lacZ* gene in KOS-1 is detected in only a small percentage of the productively infected neurons is an unusual result since DNA amplification usually results in an increased pattern of gene expression. This suggests an unusual form of regulation of the LAT promoter expression which differs greatly between acutely and latently

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pJA-1:
Pst I .....TATAAAAGCGGGGGCGCGCCGTGCCGATCGCCGGTGGTGCGA
Tata Box Transcription Start

[AAGACTTTCGGGGCGCGTCCGGGTGCCCTCGAGGTGCGACGGTATCGAT] AAGCTT
[sequence in brackets is deleted Xho I Sal I Cla I Hind III
from pWAG-15].

pWAG-15: * * *
Pst I .....TATAAAAGCGGGGGCGCGCCGTGCCGTACGCCGGTGGTGCGAAAGCTT
Tata Box Transcription Start of globin Hind III

FIG. 1. Sequences of pJA1 and pWAG15. Asterisks indicate altered nucleotides in pWAG-15.

infected neurons. Two alternative types of regulation could potentially explain this distribution of LAT promoter expression. They are not mutually exclusive. One form of regulation could be that the productively infected neurons do not have adequate levels of the transcription factors necessary for activating the LAT promoter. Alternatively, it is possible that at 4 days postinoculation, the LAT promoter is specifically downregulated by the ICP4 protein in productively infected neurons but not in phenotypically latent neurons in which ICP4 is not expressed.

To determine the extent to which LAT expression is negatively regulated in productively infected cells through the *cis*-acting element located coincident with the LAT transcription start site, we constructed a new recombinant HSV-1 altered at the LAT transcription start site to compare with KOS-1, which contains the LAT transcription start site. Experiments with these viruses as well as the plasmids from which they are derived indicate that expression of the LAT promoter is dramatically increased in cell lines and in neurons productively infected with KOS-15, the viral recombinant altered at the LAT transcription start region, compared with those infected with KOS-1. Thus, the LAT transcription start region is required for considerable downregulation of the LAT promoter expression during a productive infection.

MATERIALS AND METHODS

Plasmid construction. To construct pWAG15, a plasmid DNA containing the LAT promoter from the *Sma*I site at -863 through the *Sac*II site at +43 fused to the *lacZ* gene was subcloned as a *Bgl*II-*Hind*III fragment in SP72 (pWAG8), in which the *Bgl*II site was inserted as a linker upstream of the *Sma*I site, and the *Sac*II site was converted to an *Xho*I site, resulting in the series of adjacent sites of *Xho*I, *Sal*I, *Cla*I, and *Hind*III as part of the Bluescript polylinker. Next, an oligonucleotide was made to convert the transcription start region's sequence GATC to TACA, which is the sequence of the rabbit β -globin gene transcription start site. This oligonucleotide extended in the 3' direction for another 15 bases of LAT leader sequence before adding a *Hind*III site. Next, pJA1, which contains the same *Sma*I-to-*Hind*III DNA, was PCR amplified by using this oligonucleotide and an oligonucleotide encompassing the *Pst*I site upstream of the TATA box. The PCR product was cloned as pWAG7 and sequenced to confirm the mutagenesis of the LAT start site. Then the *Pst*I-*Hind*III fragment from this plasmid was exchanged for the *Pst*I-*Hind*III fragment of pWAG8 to generate pWAG14. The *Bgl*II-*Hind*III fragment from pWAG14 was then inserted into the *Bgl*II-*Hind*III fragment of pJA1 to generate pWAG15. In the process, 47 bases were deleted from the *lacZ* RNA leader sequence, including restriction sites for *Xho*I, *Sal*I, and *Cla*I, as these sites plus *Hind*III are present at the junction of the LAT leader and *lacZ* leader in pJA1. The sequences of pJA1 and pWAG15 are shown in Fig. 1.

All recombinant plasmids were constructed as described by Sambrook et al. (38). Construction of plasmids pGH78 (kindly supplied by Gary Hayward) and pJA1 has been previously described (28, 35). Plasmid pICP0 is an *Hpa*I-to-*Sac*I clone in pUC18 and was kindly supplied by Rozanne Sandri-Goldin.

Transient transfection assays. Rabbit skin cells transfected 2 days earlier with β -galactosidase expression plasmids (pJA1 and pWAG15) were washed, pelleted, resuspended in phosphate-buffered saline (PBS), and lysed by repeat freeze-thaw cycles. Ten microliters of transfection lysate was then added to 90 μ l of chlorophenol red- β -D-galactopyranoside (CPRG) buffer (50 mM KPO₄, 1 mM MgCl₂, 5 mM CPRG), incubated for 3 h at 37°C, and assayed for A₅₇₃ as instructed by the manufacturer (Boehringer Mannheim) for CPRG.

FIG. 2. Transient transfections demonstrating ICP4 downregulation of the LAT promoter. In panel A, the LAT promoter containing the LAT transcription start site, pJA1, was transfected into rabbit skin cells by itself or with an ICP4- or ICP0-containing plasmid, and the levels of relative β -galactosidase activity from these three transfections were determined. In the cells in which pJA1 was transfected alone, an equal amount of pBS, a Bluescript plasmid with no insert, was added. In panel B, plasmid pWAG15, which contains the β -globin transcription start site instead of the LAT start site, was used.

FIG. 3. DNA structures of two recombinant HSV-1 variants in which an HSV-1 DNA fragment containing the LAT promoter placed immediately 5' of the *lacZ* gene was inserted into the KOS strain of HSV-1 at the gC locus. KOS-1 (top) contains the LAT promoter (Pr) and the LAT transcription start site fused to the leader of the *lacZ* gene. In KOS-15 (bottom), the LAT transcription start site has been replaced by the β -globin start site.

Construction of recombinant viruses. The recombinant viruses used in this study were designated KOS-1 and KOS-15. The structure of KOS-1 has been previously described (28). To create KOS-15, KOS strain DNA was cotransfected with plasmid DNA (pWAG15), and recombinants were screened by dot blot hybridization using ^{32} P-labeled *lacZ* sequences as probes. Positive viral recombinants were plaque purified and screened three additional times, and the structures of the final viral recombinants were confirmed by Southern blot hybridization.

Productive infection of tissue culture cells. Culture plates (100-mm diameter) confluent with rabbit skin cells were infected with KOS-1 or KOS-15 at a multiplicity of infection of 1.0. Cells were harvested 8 h postinoculation, pelleted, resuspended in 100 ml of PBS, and assayed for β -galactosidase expression by using the CPRG protocol detailed above for the transient transfection assays.

Animal inoculation and tissue preparation. Female Swiss Webster mice, 4 to 6 weeks of age, were used in all experiments, and bilateral footpad or corneal inoculations were

TABLE 1. Acute in vivo infection with KOS-1 and KOS-15: analysis of dual immunofluorescent staining

Ganglion	Virus	No. positive ^a	
		HSV antigen only	HSV antigen + β -galactosidase
Dorsal root	KOS-1	3,395	44
	KOS-15	4,088	869
Trigeminal	KOS-1	7,292	15
	KOS-15	4,019	821

^a Average number of positive neuronal profiles (sections of neurons as viewed in 6- μ m sections) per animal in the infected sensory ganglia of experimental mice. For all experiments, every fifth tissue section from the pooled ganglia of five mice was analyzed.

made as described previously (21, 27). Four days postinoculation, mice were sacrificed by carbon dioxide inhalation followed by cardiac perfusion with PBS. Tissues were fixed in situ by cardiac perfusion with 4% paraformaldehyde in PBS followed by a 1-h en bloc immersion of dissected ganglia in this same fixative. Fixed ganglia was then equilibrated in 30% sucrose, frozen in liquid nitrogen, sectioned serially in a cryostat, and stored at 20°C. For dual immunofluorescent labeling for viral antigen and β -galactosidase, every fifth tissue section was defrosted, incubated in 3% normal goat serum in PBS, and then incubated in rabbit anti- β -galactosidase anti-serum (Cappel) for 1 h at room temperature. The tissue sections were then briefly washed in PBS with 1% NGS, incubated with a biotinylated goat anti-rabbit antiserum (Vector) for 35 min at room temperature, briefly washed again, and incubated for 40 min at room temperature with rhodamine 600-avidin D (Vector). The tissue was washed again, incubated with rabbit biotinylated anti-avidin D (Vector), rewashed, and incubated in PBS with 10% normal rabbit serum. A final incubation with rhodamine 600 D-rhodamine (Vector) and fluorescein isothiocyanate-labeled rabbit anti-HSV was carried out for 40 min at room temperature and followed by a final wash with 1% normal goat serum in PBS. The stained sections were then covered with glass coverslips by using Vectashield mounting medium and examined with a Nikon Microphot-5A microscope and appropriate filters.

RESULTS

Effect of the LAT transcription start site region on down-regulation of the LAT promoter. To compare the effects of ICP4 protein on constructs containing the LAT transcription start site and the altered transcription start site, a series of transfection experiments was carried out in rabbit skin cells. Plasmid pJA1 alone yielded a moderate level of β -galactosidase expression when transfected into rabbit skin cells. Cotransfection of pJA1 with a plasmid encoding the ICP4 gene resulted in significantly less β -galactosidase expression than with pJA1 alone (Fig. 2A). As a control, cotransfection of a plasmid bearing the ICP0 gene transactivated rather than repressed pJA1. These results suggest that the ICP4 protein downregulated pJA1 at the LAT transcription start site as described by Batchelor et al. (2). By contrast, a cotransfection of plasmid pWAG15 (in which the LAT start site was mutated to the β -globin start site) with the plasmid encoding ICP4 resulted in a twofold stimulation of β -galactosidase expression rather than repression. Cotransfection of pWAG15 with a plasmid bearing the ICP0 gene resulted in a substantial transactivation of pWAG15 (Fig. 2B). Transfection of

FIG. 4. ICP4 downregulation of the LAT promoter, determined by productive infection of rabbit skin cells in vitro. Rabbit skin cells were infected at a multiplicity of infection of 1 for 8 h with either KOS-1 or KOS-15. Cells were harvested, and relative β -galactosidase activities from KOS-1-infected cells (A) and from KOS-15-infected cells (B) were determined.

FIG. 5. Immunocytochemistry of dorsal root ganglia. Shown are representative tissue sections of mouse dorsal root ganglia 4 days after footpad inoculation with the HSV construct KOS-15 (A and B) or KOS-1 (C and D). The tissues were immunocytochemically stained for both HSV antigens (A and C) and β -galactosidase (B and D) as described in the text. Note that in the KOS-15-infected tissue, three of the neuronal profiles that stain for HSV antigen (A; short arrows) also stain for β -galactosidase (B; short arrows). In the KOS-1-infected tissue, none of the HSV antigen-positive neuronal profiles (C) stain for β -galactosidase (D), despite adjacent infected neuron which stains for β -galactosidase only (long arrows). Bar = 50 μ m.

pWAG15 by itself gave levels of expression similar to those obtained with pJA1 alone.

The binding site for ICP4 is well documented in several studies (11, 12, 22, 29). It has been successfully shown that repression of ICP4 gene transcription is due to ICP4 binding at the ICP4 transcription start site (29–32, 35). The start site of the LAT promoter is a five-of-six-base match with this consensus sequence, and we have confirmed that ICP4 does indeed bind to the LAT cap (data not shown). A more complete analysis of the biochemistry of ICP4 binding to the LAT cap is presented in a report by O'Hare and colleagues (2). For the present study, it is sufficient to note that removal of the LAT transcription start region from pJA1 and replacement with a β -globin transcription start region yields a plasmid which no longer contains an ICP4 binding site at the transcription start site and which is transactivated by ICP4 rather than repressed.

Effect of the LAT transcription start site region on LAT promoter-driven gene expression in productively infected cells in vitro. To compare the effects of the LAT transcription start site on LAT promoter activity in the context of the virus, we studied the activity of the LAT promoter in two different KOS strain recombinant viruses, one containing pJA1 and one containing pWAG15. The recombinant virus KOS-1 had been previously constructed by inserting plasmid pJA1 into the wild-type KOS strain by site-specific recombination (28). This virus contains an 863-bp portion of the LAT promoter, including the LAT transcription start site, fused to the *lacZ* gene and inserted into the virus at the gC locus. The recombinant virus, KOS-15, is identical to KOS-1 with the exception that the region around the LAT transcription start site has been

replaced by PCR mutagenesis to contain the β -globin start site, and the leader RNA in front of the *lacZ* gene is shortened slightly. The gross DNA structures of KOS-1 and KOS-15 are illustrated in Fig. 3; the specific sequences which were altered in pWAG-15 are shown in Fig. 1. As stated above, transfections of each plasmid by itself gave similar levels of β -galactosidase expression, and the plasmids were very similar in their responses to ICP0 transactivation. They differed only in their responses to ICP4 plasmid cotransfection.

Eight hours after inoculation of rabbit skin cells in culture with either KOS-1 or KOS-15, LAT promoter activity was measured by a chromogenic assay for β -galactosidase. We reasoned that by 8 h postinfection, the overproduction of ICP4 should result in a prolonged period of downregulation of transcription of the *lacZ* gene in KOS-1-infected cells. As shown in Fig. 4, rabbit skin cells productively infected with KOS-1 produced 8- to 10-fold lower levels of β -galactosidase compared with cells infected with KOS-15, in which the LAT transcription start site was replaced with the β -globin transcription start site.

Effect of the LAT transcription start site on LAT promoter-driven expression in acutely infected neurons. We have previously shown that infection of mouse dorsal root ganglia with KOS-1 for a period of 4 days results in an unusual pattern of β -galactosidase expression (28). By dual immunofluorescence microscopy, we observed that most of the productively infected neurons, expressing viral antigen, were not positive for β -galactosidase expression. By contrast, the neurons expressing β -galactosidase were almost all negative for viral antigen expression. In the latter case, this can be understood: at latent

FIG. 6. Immunocytochemistry of trigeminal ganglia. Shown are representative tissue sections of mouse trigeminal ganglia 3 days after ocular inoculation with the HSV construct KOS-15 (A and B) or KOS-1 (C and D). The tissues were immunocytochemically stained for both HSV antigens (A and C) and β -galactosidase (B and D) as described in the text. Note that in the KOS-15-infected tissue, three of the neuronal profiles that stain for HSV antigen (A; short arrows) also stain for β -galactosidase (B; short arrows). In the KOS-1-infected tissue, none of the HSV antigen-positive neuronal profiles (C) stain for β -galactosidase (D), despite adjacent infected neuron which stains for β -galactosidase only (long arrows). Bar = 50 μ m.

times, a similar number of neurons were expressing only β -galactosidase and not viral antigen. Therefore, neurons expressing only β -galactosidase at 4 days might be called phenotypically latent. But why is β -galactosidase not expressed in neurons productively infected? To determine if this could be caused by the presence of the LAT start site in KOS-1, we infected mouse dorsal root ganglia for 4 days with either KOS-1 or KOS-15 and sectioned the ganglia. Antisera to viral antigen and to β -galactosidase were used in a dual immunofluorescence study of these sections, and the results are presented in Table 1. In KOS-1-infected mice, only 1.2% of HSV antigen-positive neurons stain for β -galactosidase. By contrast, in KOS-15-infected mice, 18% of the HSV antigen-positive neurons coexpress β -galactosidase (Fig. 5). Similar findings were observed in trigeminal ganglia at 3 days after ocular inoculation with KOS-1 and KOS-15 (Fig. 6).

DISCUSSION

Previous studies showed that the pathways of acute and latent gene expression diverge early in the course of acute neuronal infection (23, 28, 39–42, 50). An interesting finding in these studies, and one which helped to establish the principle of separate infections, is that the LAT promoter is relatively inactive in neurons undergoing a productive infection with HSV-1. To determine the extent to which ICP4 repression of the LAT promoter activity causes this early divergence of acute and latent gene expression, we have used two recombinant HSV-1 variants that each have LAT promoter-*lacZ* gene

fusions inserted at the gC locus. These two viruses differ mainly in the transcription start sequences for the LAT promoter-*lacZ* gene fusions, one containing the LAT transcription start site (KOS-1) and the other containing the β -globin transcription start site (KOS-15).

LAT is the sole detectable viral transcript found in sensory neurons latently infected with HSV-1 (5, 36, 37, 46, 49). The expression of this transcript in neurons is not dependent on viral protein expression or DNA replication (23, 50). In fact, LAT is absent from the vast majority of acutely infected neurons by day 4 postinoculation (28). Ho and Mocarski found that β -galactosidase expression driven by the LAT promoter could not be detected in acutely infected peripheral tissues (19). LAT expression in productively infected culture cells may be 1,000-fold less than in latently infected neurons (53). These observations suggest that stringent controls are placed on the expression of LAT during a productive infection.

Batchelor et al. showed that ICP4 downregulates transcription driven by the LAT promoter in transient transfection assays (2). Furthermore, they identified the region encompassing the LAT transcription start site as the *cis*-acting DNA sequence through which ICP4 affects the LAT promoter. The LAT transcription start site has a high degree of similarity to the ICP4 consensus DNA binding site. In most instances, ICP4 acts as a transactivator of gene expression (9, 14, 30–34). However, it downregulates expression of its own transcription through an ICP4 binding site located at its own transcription start site (12, 29, 32, 35). We confirmed that ICP4 downregu-

lates LAT promoter transcription through a *cis*-acting element coincident with the LAT transcription start. Plasmids containing the normal LAT transcription start site region are down-regulated by cotransfection with an ICP4-expressing plasmid but not with an ICP0-expressing plasmid, and a plasmid with the β -globin transcription start site is not downregulated by ICP4. Similar results are observed in infections of rabbit skin cells with recombinant viruses containing these two plasmid DNAs. KOS-1-infected cells show 10-fold less expression of the β -galactosidase gene product than KOS-15-infected cells. Infection of either murine dorsal root ganglia or trigeminal ganglia *in vivo* also reveals that the LAT transcription start site plays a substantial role in repression of the LAT promoter activity *in vivo*. Thus, expression of the β -galactosidase gene increased dramatically, as did the percentage of cells coexpressing both viral and β -galactosidase antigen, when the LAT start site was replaced. Since neither the LAT (GATC) nor the β -globin (TACA) transcription start site is a good match for an initiator element (CATT), it is unlikely that the difference in expression is due to a difference in the ability of either to function as an initiator (20).

We also note that although replacement of the LAT transcription start site dramatically increases LAT promoter activity, the percentage of neurons which coexpress viral antigen and β -galactosidase is significantly less than 100%. The reason for this is not clear. However, it might be that viral antigen is more easily detected than β -galactosidase, either because viral antigen is present in greater quantity or because the assay for viral antigen is more sensitive. Alternatively, it could be that there is a class of neurons which can be productively infected but which have relatively low levels of transcription factors for the LAT promoter.

In these studies, we have examined the effect of the LAT transcription start site on the activity of the LAT promoter *in vitro* and *in vivo*. The *in vivo* studies are particularly significant because LAT promoter activity is high only in neurons *in vivo*. In cells in culture or in plasmid transfections, the promoter is relatively inactive. The results of our studies suggest that the transcription start site has a very strong effect *in vivo*, despite the high activity of the LAT promoter in these neurons. In neurons which are productively infected, it is clearly a dominant regulatory element. By contrast, in neurons which are latently infected, the LAT transcription start site probably has no effect at all, since there is no ICP4 protein available to compete with activation of the promoter by transcription factors.

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