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The Activity of the Pseudorabies Virus Latency-Associated Transcript Promoter Is Dependent on Its Genomic Location in Herpes Simplex Virus Recombinants as well as on the Type of Cell Infected

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As do many other alphaherpesviruses, pseudorabies virus (PRV) transcribes a limited portion of its viral genome in latently infected neurons during latency. The sequence of the PRV latency-associated transcript (LAT) is bounded on its 5′ end by a putative promoter region which contains sequence elements similar to those characterized for the herpes simplex virus (HSV) LAT promoter. Using the bacterial β-galactosidase gene as a reporter, we have assayed PRV LAT promoter activity in the genomic environment in recombinant HSVs. The PRV LAT promoter–β-galactosidase reporter gene was recombined into the terminal and internal long repeat regions (R_L regions), replacing the normal HSV LAT promoter, the cap site, and the first 60 bases of the primary transcript. When recombined into the R_L region, appreciable reporter gene expression was observed following infection of two cell lines of neuronal origin; little or no activity was seen with these recombinants following infection of rabbit skin or mouse embryo fibroblasts. No significant expression was seen when the promoter was recombined into the gC locus in the long unique region in any of the cell types utilized. Such results suggest that the PRV latency promoter contains neuronal cell-specific elements and that the HSV R_L region provides an appropriate genomic environment for the manifestation of that specificity.

The neurotrophic or alphaherpesviruses establish latent infection in sensory neurons; basic aspects of such latent infections are described in a number of recent reviews (1, 24, 31, 32). Four of these viruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), pseudorabies virus (PRV), and bovine herpesvirus type 1 are known to transcribe a limited portion of the viral genome during latency; in the most extensively studied representative, HSV-1, this latent-phase transcription has been shown to have a role in efficient in vivo reactivation from the latent-state systems (3, 13). It also has a role in efficient in vitro explant-induced reactivation of virus from latently infected murine ganglia (18, 30). It may also have a role in the efficiency of establishment of latent infection in in vitro murine models (6, 26); however, the relationship between these two roles is not yet established.

Despite uncertainty as to mode of action, it has been established that the HSV-1 latency-associated transcripts (LATs) are controlled by a canonical polymerase II promoter which is transcriptionally active in latently infected neurons and which has been shown to exhibit some features of neuronal specificity in transient-expression assays with cultured cells (2, 33–35). This promoter is located approximately 1,700 bases downstream (3′) of the α0 (ICP0 or $V_{MW}110$) transcript terminus, and RNase protection experiments have shown that the LAT primary transcript initiates just 3′ of a TATA box (8). Potential control elements which could have a role in LAT's expression during latent infections include a canonical cyclic AMP response element (CREB), CAT box, Sp1 binding sites, and a strong ICP4 (α4) binding site which is involved in its autoregulation (18, 19, 34; also reviewed in references 24 and 32).

The PRV LAT and its corresponding promoter elements have not been so well characterized; however, the transcript appears to have some features reminiscent of the HSV-1 LAT. High-resolution in situ hybridizations with short DNA probes and latently infected porcine ganglia have located the 5′ end of the PRV latency transcript just downstream of a potential promoter containing sequence elements similar to those present in the HSV-1 LAT promoter (22, 23). Such a location is consistent with the results of cDNA mapping studies reported by Cheung (4). The sequence for the Ka strain of PRV extending from −420 to +66 relative to this putative cap site is shown in Fig. 1; nominal promoter control elements along with the position of a potential CREB and PRV autoregulatory immediate-early protein (PRV-IEP) binding site are indicated.

The general similarity of sequence elements between the well-characterized HSV-1 LAT promoter and those in the putative PRV LAT promoter at analogous locations relative to LATs suggests functional similarity. In order to verify the identity of the PRV LAT promoter and to investigate potential neuronal specificity, we generated reporter constructs containing differing extents of the potential PRV LAT promoter linked to the bacterial β-galactosidase gene and recombined it into two locations in the HSV genome. We then examined patterns of reporter gene expression following infection of cultured cells of various origins. These patterns of expression suggest that the properties first described for the HSV-1 LAT promoter are also seen with PRV. Since similar patterns have been described from the bovine herpesvirus LAT promoter (16, 17), we conclude that they are probably general for those alphaherpesviruses expressing LATs.

The high-resolution in situ hybridization analysis of Priola and Stevens (23) placed the 5′ limit of the PRV LAT in a

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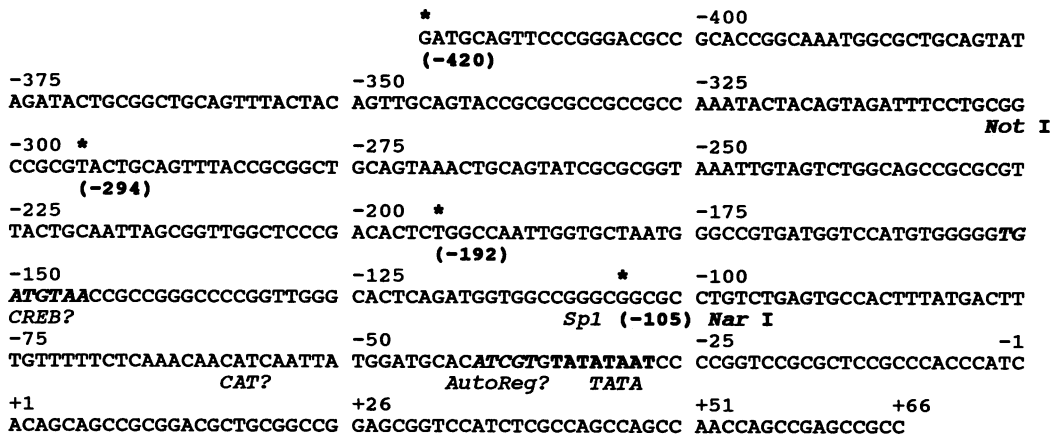

FIG. 1. Sequence of the PRV strain Ka latent-phase transcript promoter. Data to the *Not*I site were determined by Priola and Stevens (23). Some of the specific deletions used in the paper as well as some potential control elements are indicated. The transcript start site is determined from data presented in this paper. *AutoReg*?, putative PRV autoregulatory IEP binding site.

*Nar*I-*Bam*HI subfragment of the *Bam*HI fragment 6 of the Ka strain. We confirmed the fact that this DNA contains an actual transcription start site, indicated in Fig. 1, in the experiments described below. In order to investigate the PRV latency promoter, we subcloned a 1.3-kbp fragment extending from the *Xho*I site located approximately 900 bp upstream of the transcript cap site to the *Bam*HI site about 400 bp downstream into a pUC18-based vector. A 3′ deletion to +66 relative to this cap site was made by opening the plasmid at the *Bam*HI site, digestion with *Bal* 31 enzyme, and then ligation of a *Hin*dIII linker by using methods described previously (9, 10, 12, 14). Separate 5′ deletions were made by cleavage of the parental clone at the *Xho*I site, digestion with *Bal* 31, and ligation in *Sal*I linkers. Nested 5′ deletions extending to +66 were constructed by combining 5′ and 3′ deletions at the *Nar*I site at −105; other deletions were constructed by digestion with either *Sal*I or *Nar*I and insertion of appropriate linkers. The sequences of all deletions were confirmed by dideoxy sequencing (25).

The activity of the PRV promoter-controlled reporter gene constructs was assayed by transient-expression assays, and some activity was seen in transfected early-passage murine neuroblastoma cells (data not shown). It is well documented, however, that transient-expression assays do not necessarily reflect the expression patterns observed in vivo (12, 15, 27); therefore, we examined the activity of this promoter within the context of a viral genome. In order to take advantage of the recombination systems currently employed in our laboratory, we generated recombinant viruses containing portions of the PRV latency promoter controlling β-galactosidase by cotransfecting wild-type (wt) HSV-1 (17*syn*$^+$) DNA with appropriate recombination plasmids by using methods described in previous publications (7, 9, 11, 12, 14). Most recombinants were into the HSV-1 terminal and internal long repeat regions (R_L) replacing the LAT promoter, cap site, and first 60 bases of the primary LAT. The −900/+66, −420/+66, and −294/+66 promoters were also recombined into the gC locus to assess positional effects on promoter activity. These sites are shown schematically in Fig. 2; Southern blots of purified virus isolates verifying the absence of contaminating wt parents are also shown in Fig. 2. Two independent recombinants (each from an independent transfection) were isolated for each construct and assayed. The sequence of the promoters inserted was confirmed by DNA sequencing. Recombination into the gC location was accomplished by using the recombination vector described (9, 11); for the R_L recombinants, we used a recombination plasmid containing HSV sequences entirely within the R_L in order to optimize the formation of recombinant viruses containing the desired reporter element in both the terminal R_L and the internal R_L. This vector was made by cloning the *Nco*I-*Kpn*I fragment (bases 116,919 to 119,966 of the HSV-1 17*syn*$^+$ genome [20, 21]) into a pUC18-based vector, removal of DNA to base 117,587 by *Bal* 31 digestion at the *Nco*I site, and insertion of a *Sal*I linker. Next, a synthetic *Xba*I-*Xho*I-*Bam*HI adaptor was used to replace the 221-bp *Eco*RV-*Pst*I fragment spanning bases 118,640 to 118,861. This removed 160 bp of the proximal HSV-1 LAT promoter as well as the first 60 bases 3′ of the cap site of the primary transcript. Following this, the *Kpn*I site was converted to a *Sal*I site by using an appropriate linker, the *Sal*I-*Sal*I fragment was cloned into pGEM-1, and the approximately 4.1-kbp *Sal*I-*Bam*HI DNA fragment containing the appropriate portion of the PRV latency promoter, the β-galactosidase gene, and the simian virus 40 polyadenylation sequences were ligated into the *Xho*I-*Bam*HI sites of the recombination plasmid. The promoter sequences controlling β-galactosidase and flanking HSV sequences were excised from the plasmid by *Sal*I digestion for cotransfection with wt DNA.

In order to characterize the activity of the PRV LAT promoter, various recombinant viruses were used to infect 35-mm (10^3-mm^2)-diameter plates containing approximately 10^6 rabbit skin, murine neuroblastoma (Neuro-2A), human neuroblastoma (IMR-32), or mouse embryo fibroblasts cells with a multiplicity of infection of 1 PFU per cell. Cells were harvested 24 h following infection, and aliquots were assayed for enzyme activity. Two recombinant viruses, FLA5 and dUTPase/LAT, were used as controls. The former contains 50 bases of the HSV-1 γ U_L38 promoter controlling β-galactosidase in the same location of the R_L (11), while the latter contains the HSV-1 β dUTPase (U_L50) promoter in this location (14). Typical data are shown in Table 1; it is clear that, unlike the recombinants containing the HSV-1 promoters, all variants of the PRV latency promoter were essentially inactive during lytic infection of rabbit skin cell or mouse embryo fibroblast cells whether present in the R_L or the gC location of the genome. In contrast, all PRV latency promoters recombined into the R_L expressed β-galactosidase in lytically infected Neuro-2A cells. Infection of IMR-32 cells with the

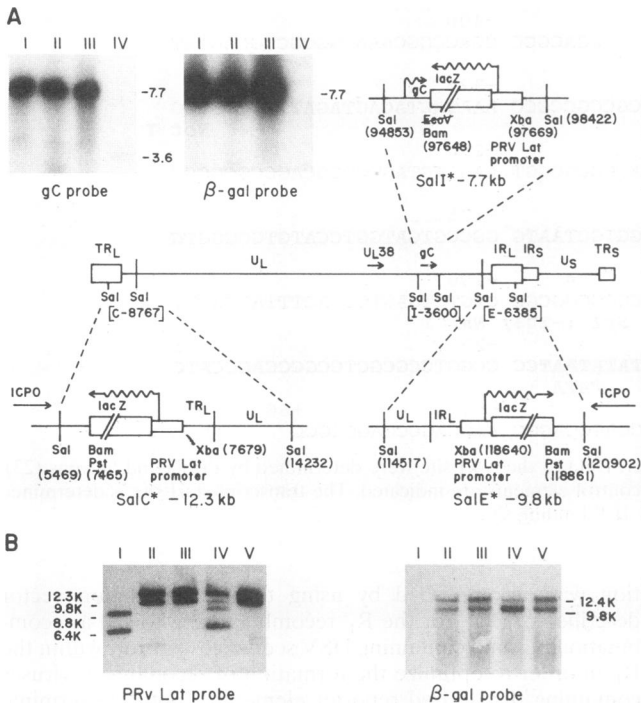

FIG. 2. Sites of recombination of the PRV latent-phase promoter controlling β-galactosidase in the HSV-1 genome. (A) Recombination in the gC location. Lanes i, ii, and iii, Southern blots of DNA from separate isolates of the −294/+66 gC recombinant digested with *Sal*I and hybridized with either a gC- or β-galactosidase-specific probe as previously described (11); lane iv, the 3.6-kb *Sal*I band obtained by digestion of wt viral DNA. (B) Insertion into the R_L location. Lanes i, Southern blot of a *Sal*I digest of wt viral DNA; lanes ii, an isolate of the −420/+66 recombinant; lanes iii, the −294/+66 recombinant; lanes iv, the −192/+66 recombinant; lanes v, the −105/+66 recombinant. The PRV LAT probe spanned bases 117,587 to 119,966 within the HSV R_L (21). Note that the −192/+66 recombinant was not pure at this stage and was subjected to further plaque purification. Purity of all recombinant viruses was confirmed by PCR analysis as previously described (12).

−298 PRV promoter construct resulted in reporter gene activity comparable to that seen upon infection of Neuro-2A cells (data not shown).

The lack of expression of PRV LAT promoter-controlled β-galactosidase by gC recombinants in all cell types tested suggests that the expression of the PRV latency promoter in neuronal cells is strongly dependent on its location within the HSV genome. The fact that even 900 bases of sequence upstream of the putative cap site is not sufficient to allow expression suggests that specific HSV sequences 5′ of the *Eco*RV site at base 118,640 (and in an equivalent location in the terminal R_L) are important in the neuronal specificity seen here. The lack of activity of the PRV/R_L promoter recombinants in mouse embryo fibroblasts demonstrates that the activity seen in the murine neuroblastoma cells is cell type—not cell species—specific.

While we have shown elsewhere that β-galactosidase activity expressed by recombinant viruses in infected cells is a reliable measure of levels of mRNA expression (12), the data shown here cannot preclude the possibility of expression of a β-galactosidase-containing transcript expressed by some cryptic HSV promoter located upstream of the PRV promoter insertion. We verified that the enzyme expression seen was the

TABLE 1. β-Galactosidase activity expressed in cells infected with recombinant HSV[a]

Virus	Cells		
	Rabbit skin	Neuro-2A	MEF[b]
17*syn*$^+$	20 ± 5	20 ± 5	40 ± 5
FLA5[c]	543 ± 143	543 ± 100	280 ± 70
dUTPase/LAT[d]	890 ± 50	1,037 ± 50	ND[e]
−420/+66	50 ± 15	296 ± 50	50 ± 10
−294/+66	35 ± 10	445 ± 100	45 ± 10
−192/+66	30 ± 10	248 ± 50	ND
−105/+66	30 ± 15	200 ± 50	ND
−294/+66(gC)	10 ± 5	50 ± 10	ND
−420/+66(gC)	20 ± 5	30 ± 10	ND
−900/+66(gC)	25 ± 5	30 ± 10	ND

[a] β-Galactosidase activity was assayed by the *o*-nitrophenol method as previously described (9, 10, 14). Activity is expressed in units of enzyme activity, where 1 U of β-galactosidase cleaves 1 nmol of *o*-nitrophenol phosphate per min at 30°C, and 1 nmol of product (*o*-nitrophenol) has an A_{414} of 0.0045. For measuring β-galactosidase activity expressed by each recombinant virus, cells of each indicated type were infected with specific recombinant virus as indicated or with wt 17*syn*$^+$ virus at a multiplicity of infection of 1 PFU per cell. Cell extracts were prepared and assayed for β-galactosidase activity 24 h postinfection. Harvested cells were lysed by three freeze and thaw cycles in a buffer containing 0.25 M sucrose, 10 mM Tris · HCl (pH 7.4), and 10 mM EDTA, and 50-μl aliquots were assayed. The values given were derived from the averages and standard deviations of three separate experiments with different preparations of recombinant viruses.

[b] MEF, mouse embryo fibroblasts.

[c] See text and reference 11.

[d] See text and reference 14.

[e] ND, not determined.

result of specific PRV LAT promoter-controlled transcription as follows. RNA was isolated from Neuro-2A cells at 8 h following infection with the −294/+66 R_L recombinant by the guanidinium isothiocyanate method, and poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography (9). Hybridization of 3 μg of this RNA with the 368-base opposite-sense RNA probe generated by in vitro transcription with T7 RNA polymerase (Promega) followed by RNase T_1 digestion generated a major protected species approximately 66 bases in length and a smaller amount of a 72- to 75-base product (Fig. 3A). This confirms the initiation of a transcript approximately 30 bases downstream of the TATA box homology identified by Priola and Stevens (23) and shown in Fig. 1. Additional evidence for PRV LAT-driven transcription was provided by Northern (RNA) blot hybridization. Poly(A) (3 and 6 μg)-containing RNA was size fractionated on a formaldehyde-containing agarose gel by electrophoresis and blotted onto nitrocellulose membranes as previously described (9). Hybridization of this blot with β-galactosidase-specific nick-translated DNA probe demonstrated a 3.6-kb transcript—again, the expected size for the inserted reporter gene (Fig. 3B).

The results described here demonstrate that PRV latency promoter previously identified by in situ transcript mapping has properties consistent with neuronal specificity. We are currently in the process of investigating the expression of the reporter gene in sensory ganglia latently infected with the recombinant viruses described here; however, the present data provide some basic information concerning latent-phase gene expression by alphaherpesviruses. First, our data confirm some general structural similarities between the PRV and HSV-1 LAT promoters; such similarities between LAT promoters of these two viruses and that of bovine herpesvirus type 1, which has been partially characterized by Rock and colleagues (16, 17), can also be inferred. Second, the data confirm our earlier conclusion that the actual genomic position of functional

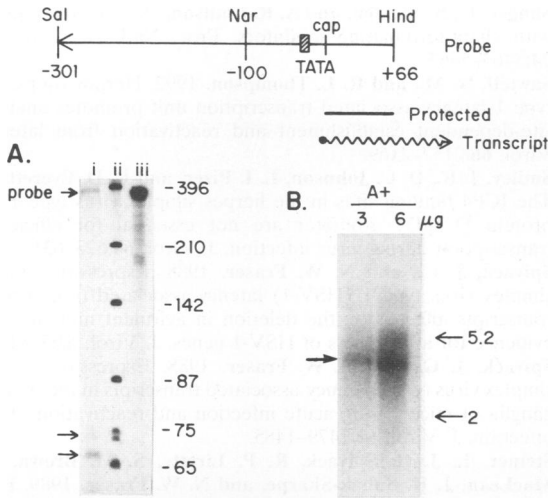

FIG. 3. Chimeric β-galactosidase mRNA expressed in Neuro-2A cells infected with the −294/+66 R_L recombinant virus. (A) RNase protection assays. A total of 5 μg of poly(A)-containing infected-cell RNA was hybridized with the antisense RNA probe spanning −301 to +66, as shown above. The protected species are shown with arrows in lane i. Lane ii, migration of a DNA size marker; lane iii, migration of the undigested probe. (B) Northern blot analysis of poly(A)-containing RNA (A+) RNA isolated from infected Neuro-2A cells. The position of 5.2- and 2-kb rRNA migrations are indicated by arrows, and the position of the 3.6-kb β-galactosidase-containing transcript is indicated by an arrow on the left. Details of fractionation and blotting procedures have been described (7, 11).

promoter elements within the HSV genome can have significant influence on activity (11). In regard to the latter point, the present data also confirm our earlier inference from transient-expression assays that sequence elements in the HSV R_L spatially removed from the LAT promoter itself are important in neuronal specificity (7). Thus, even 900 bases of upstream PRV sequences is not adequate for reporter gene expression within the gC location in the U_L, while as little as 104 bases of the PRV promoter is sufficient for measurable activity in the R_L—this suggests that the upstream boundary of the PRV LAT promoter lies within 104 bases from the transcript start site.

It is of interest to consider why the core PRV latency promoter is not active in lytic infection in rabbit skin cells despite its having features of a canonical polymerase II promoter. The presence of the ICP4 autoregulatory binding site near the HSV LAT cap site has been cited as a factor in down regulating this promoter during lytic HSV infection (28, 29); however, the HSV LAT promoter is clearly active at a measurable level in lytic infection in most cultured cells (reviewed in reference 24). The homologous autoregulatory binding site in the PRV promoter could have a similar but more tightly regulated function, but despite Cheung's inability to isolate LAT-specific cDNAs from cells lytically infected with PRV (5), Priola et al. identified homologous transcripts from both lytically and latently infected cells (22). A potentially more interesting possibility is that Neuro-2A (and, by inference, other neuronal) cells provide some additional transcription factor necessary for core PRV latent-phase promoter function in the R_L region even in the presence of the generalized transcriptional activation by HSV ICP4 and other immediate-early regulatory proteins. A similar factor or factors may also be involved but not strictly required during HSV infection of such cells, since we have previously described differences in expression of HSV LATs in lytically infected Neuro-2A cells compared with that in rabbit skin cells (7).

No matter what the mechanistic basis for the effect of genomic location on the activity of the PRV LAT promoter, it clearly demonstrates and confirms our earlier conclusions that the actual location of reporter gene constructs in the recombinant genome has a nontrivial role in levels of gene expression (11). Such effects must be taken into consideration when recombinant reporter constructs are used to assay gene activity in vivo and in vitro.

Nucleotide sequence accession number. The sequence of the PRV strain Ka LAT promoter has been submitted to GenBank under accession number U04326.

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