The latency-associated transcript promoter of pseudorabies virus directs neuron-specific expression in trigeminal ganglia of transgenic mice

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INTRODUCTION

Pseudorabies virus (PRV), the causative agent of Aujeszky’s disease in pigs, is a member of Alphaherpesvirinae subfamily (Roizman, 1990). Alphaherpesviruses, such as herpes simplex virus type I (HSV-1), varicella-zoster virus (VZV) and PRV, have a similar gene arrangement and their gene products share considerable amino acid sequence homologies. PRV causes severe disease in piglets leading to latent infection in all surviving pigs (Rziha et al., 1999). As a result of environmental or physical stress, latent PRV may be reactivated and shed, resulting in the spread of PRV infection to other animals. Thus, pigs that have once undergone a PRV infection are potential sources of the virus for their entire lifetime. PRV infection, therefore, inflicts serious losses on the swine industry worldwide.

During PRV latency, PRV DNA is retained in neurons of the trigeminal ganglion, and gene expression is restricted to a small region of the viral genome (Cheung, 1989a). RNA, termed latency-associated transcript (LAT), is synthesized in the opposite direction relative to IE and EP0 gene transcription (Fig. 1A). Several sizes of LAT (8-4, 4-5 to 5-0, 2-0 and 0-95 kb) have been detected in latently infected porcine trigeminal ganglia (Cheung, 1989b; Priola & Stevens, 1991). A spliced 8-4 kb poly(A) RNA, designated large latency transcript (LLT), has been detected in the trigeminal ganglia of both latently and lytically infected swine. Although several types of LAT are stable and accumulate in latent neurons, their function remains unclear. Several pieces of evidence suggest that LAT plays some role in facilitating reactivation of HSV from latency (Leib et al., 1989; Hill et al., 1990; Block et al., 1993; Bloom et al., 1994; Perng et al., 1994; Krause et al., 1995). On the other hand, some reports suggest that LAT is involved in establishment of latency (Sawtell & Thompson, 1992; Garber et al., 1997; Thompson & Sawtell, 1997; Kramer et al., 1998). Recently, it has been reported that LAT provides an anti-apoptotic function resulting in the survival of neurons during establishment of and/or reactivation from latency (Perng et al., 2000; Inman et al., 2001; Thompson & Sawtell, 2001; Ahmed et al., 2002).

The regulatory mechanism of PRV LAT gene expression during latency remains unknown. Based on the available data (Cheung, 1989a; Jin & Scherba, 1999), two different sequences, LAP1 and LAP2, may function in PRV LAT gene expression as the LAT promoter. PRV LLT is initiated 34 nucleotides downstream from the first TATA box in LAP1 (Fig. 1B) (Cheung, 1991). LAP1, containing the first TATA box and three CAAT boxes, is thought to act as the first latency-active promoter. In contrast, the LAT gene is transcribed 243 nucleotides downstream from the initiation site for LLT in LAP2 (Cheung, 1989a; Jin & Scherba, 1999). LAP2, containing the second TATA box and two
GC boxes, is thought to act as a second regulator. Using transient expression assays, a 1015 bp MluI–BamHI fragment (LAP) consisting of LAP1 and LAP2 (Fig. 1B) was shown to be significantly active in both neuronal and non-neuronal cells (Cheung & Smith, 1999; Taharaguchi et al., 2002). These observations suggest that the PRV LAP may be a pan-specific promoter. However, neither of these studies identified tissues targeted for expression by the PRV LAP.

Transgenic technology has provided a means to examine the specific effect of promoter regulatory elements in vivo. In this study, we examined tissue-specific expression of the PRV LAP-CAT transgene in adult transgenic mice. Our results indicate that the PRV LAP directs neuron-specific expression in their trigeminal ganglia.

**METHODS**

**Generation of LAP-CAT transgenic mice.** Plasmid pMluI/CAT (Taharaguchi et al., 2002) was digested with KpnI and HindIII to isolate the PRV LAP-CAT transgene. The 2.7 kb KpnI–HindIII fragment (Fig. 1B) from the construct was purified, and approximately 500 copies were microinjected into the pronuclei of fertilized C57BL/6 or B6C3F1 (C57BL/6 × C3H/He) mouse embryos. These embryos were subsequently transplanted into the oviducts of pseudopregnant foster recipient mice. Genomic DNA was isolated from tail tissue, and the transgene was detected by PCR analysis.
The PCR reaction was carried out in a 50 μl final volume comprising 1× PCR buffer (Takara), 1.5 mM MgCl₂, 200 μM each dNTP, 0.25 mM each primer and 2.5 U Taq DNA polymerase (Takara). Thirty cycles of amplification were carried out at 94°C for 45 s, annealing at 58°C for 30 s and extension at 72°C for 90 s. The PCR products were fractionated on a 1.5% agarose gel. Transgenic copy number was estimated by comparing the band intensity of transgenic mouse DNA with those of control DNA. The DNA samples (10 μg) were digested with BamHI, fractionated on 1% agarose gel and transferred to Hybond-N+ membranes (Amersham) by capillary blotting. DIGoxigenin (DIG)-labelled DNA probes for detection of the transgene were derived from the CAT gene using the specific primers and a PCR DIG probe synthesis kit (Roche). Hybridization and detection of the transgene were performed as described previously (Ono et al., 1999). The transgenic copy numbers were measured by scanning the membrane using a Luminexcent image analyser (LAS-1000 plus; Fuji Film) and then by densitometric analysis of images using Image Gauge version 3.5 software (Fuji Film).

**Detection of CAT expression in mouse tissues.** Mouse tissues were suspended into 30 vols of cold lysis buffer (137 mM NaCl, 10% glycerol, 0.5 mM Na₂VO₄, 1% NP40, 20 mM Tris/HCl, pH 7.5) containing 0.5 mM PMSF and homogenized with a high-speed homogenizer (Ultra-Turrax T25; IKA Labortechnik). Those homogenates were centrifuged at 12000 g for 20 min, and the supernatants were collected and stored at −70°C until analysis. Each tissue extract (approximately 150 μg in 200 μl) was used for CAT ELISA (Roche) to measure the level of CAT in tissues of transgenic mice. CAT ELISA was performed as directed by the manufacturer. For normalization, total protein concentration in each tissue extract was determined with a Bio-Rad protein assay kit.

**Western blot analysis.** Each tissue extract (400 μg) was used for immunoprecipitation with 40 μg of polyclonal sheep anti-CAT IgG (Roche) for 2 h at room temperature. Immunocomplexes were collected by centrifugation after incubating the extracts with 25 μl of Omnisorb (Calbiochem). The pellet was washed three times with PBS containing 0.05% Tween 20 and suspended in 20 μl of 2× SDS sample buffer containing 5% 2-mercaptoethanol. Samples were boiled at 100°C for 5 min and separated by 16% SDS-PAGE. Fifteen pg of recombinant CAT protein (Roche) was used for the positive control of the blot. The separated proteins were then transferred to Immobilon transfer membrane (Millipore). The membrane was treated with Buffer 1 (0.15 M NaCl, 0.1 M maleic acid, pH 7.5) containing 1% Blocking reagent (Roche). After blocking for 30 min, the membrane was washed three times with Buffer 1 containing 0.03% Tween 20 and incubated for 1 h with DIG-conjugated polyclonal sheep anti-CAT antibody (Roche) in Buffer 1 containing 1% Blocking reagent. After incubation with the primary antibody, the membrane was washed as above and incubated with alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche) for 30 min. The membrane was washed and equilibrated in Buffer 3 (100 mM NaCl, 100 mM Tris/HCl, pH 9.5). The antigen was detected using a CDP-star detection reagent (Amersham) as substrate.

**Analysis of transgene expression by RT-PCR.** Total cellular RNA was isolated from various tissues of the transgenic mice using TRIzol reagent (Gibco BRL). For RT-PCR analysis, one microgram total RNA was digested with RNase-free DNase I (Gibco BRL) to remove any contaminating genomic DNA. The cDNA was synthesized from the DNase I-treated total RNA by MMLV reverse transcriptase (Gibco BRL) using oligo(dT) as a primer. The PCR reaction for the CAT gene was carried out as described above except that amplification was performed with 15 cycles instead of 30 cycles. Control samples without reverse transcriptase were amplified in parallel to confirm the absence of genomic DNA contamination. The PCR products were fractionated on 1.5% agarose gel and analysed by Southern blot analysis. DIG-labelled DNA probes for detection of the transgene were derived from pCAT/Basic using the specific primers and a PCR DIG probe synthesis kit (Roche). Hybridization and detection of the transgene were performed as described previously (Ono et al., 1999).

**In situ hybridization.** A HindIII–XbaI fragment containing the CAT gene was cloned into pGEM–2z (Promega). Probes for sense and antisense CAT transcripts were prepared from the linearized plasmids using a DIG RNA labelling kit (Roche) according to the manufacturer’s instructions. Mouse tissues were fixed with 4% paraformaldehyde/PBS (PFA/PBS), and then embedded in paraffin. Sections (4 μm thickness) were collected onto glutaraldehyde-activated 3-aminopropyltriethoxysilane-coated slides and de-waxed in xylene before use. Tissue sections were treated with 0.2 M HCl and subsequently with 10 μg proteinase K ml⁻¹ for 15 min at 37°C. The sections were refixed in 4% PFA/PBS for 10 min and treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Riboprobes were diluted in hybridization buffer [50% formamide, 500 mM NaCl, 10 mM Tris/HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1× Denhardt’s solution, 10% (w/v) dextran sulfate, 200 μg yeast tRNA ml⁻¹, 100 μg salmon sperm DNA ml⁻¹], applied to the sections, and hybridized for 16–18 h at 50°C. After hybridization, the sections were washed twice with 0.2× SSC and blocked for 30 min at room temperature with 1× DIG blocking reagent in Buffer 1. Bound probe was detected with alkaline phosphatase-conjugated anti-DIG antibody, as per the manufacturer’s instructions (Roche).

**Effect of reactivation stimuli on LAP activity.** Transgenic mice were sacrificed and their trigeminal ganglia were dissected out and explanted into the wells of a six-well plate. They were cultured for 24 h at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 10% foetal calf serum and antibiotics. Transgenic mice were given an intravenous injection of 0.2 mg dexamethasone (Sigma) in water. Control transgenic mice were also given water without drug. Twenty-four hours after the dexamethasone injection, the trigeminal ganglia were removed. The levels of CAT in trigeminal ganglia of the transgenic mice were measured by using CAT ELISA described above.

**RESULTS**

**Generation of LAP-CAT transgenic mice**

A 1015 bp MluI–BamHI fragment (LAP) consisting of LAP1 and LAP2 is capable of efficiently driving expression of the reporter gene in both neuronal and non-neuronal cells (Cheung & Smith, 1999; Taharaguchi et al., 2002). Since this promoter controls expression of the LAT gene, activity and tissue-specificity of the promoter may be concerned with establishment of and/or reactivation from latency. To address this issue, three transgenic mouse lines containing the LAP-CAT transgene, composed of the CAT gene under the control of the 1015 bp fragment, were established. Three founder mice containing the LAP-CAT transgene were identified by PCR analysis of their tail DNA. All founders gave rise to offspring in crosses with non-transgenic littermate mice and transmitted the introduced gene in Mendelian fashion. Southern blot analysis performed on genomic DNA from these mice showed that the intensity of each transgene was different among the transgenic mouse lines (Fig. 1C). The results indicate that
each line varied in transgene copy number (68 copies for TgM16, 34 copies for TgM27 and 30 copies for TgM37).

### Table 1. CAT expression in various tissues of the LAP-CAT transgenic mouse lines

CAT expression data are means of three independent measurements and correspond to different mice in a given transgenic line.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TgM16</th>
<th>TgM27</th>
<th>TgM37</th>
</tr>
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<tbody>
<tr>
<td>Skin</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Skeletal muscles</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Heart muscles</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lung</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Small intestine</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spleen</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kidney</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>–</td>
<td>2.3 ± 0.7</td>
<td>–</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>3.0 ± 0.1</td>
<td>2.9 ± 1.3</td>
<td>–</td>
</tr>
<tr>
<td>Trigeminal ganglia</td>
<td>36.0 ± 11.5</td>
<td>9.0 ± 1.5</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>2.0 ± 0.3</td>
<td>3.4 ± 2.0</td>
<td>–</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>–</td>
<td>2.3 ± 1.2</td>
<td>–</td>
</tr>
<tr>
<td>Testis</td>
<td>–</td>
<td>2.6 ± 1.6</td>
<td>–</td>
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*< 2.0.

### Expression of the CAT gene in transgenic mice

Expression levels of the CAT gene in various tissues of each transgenic mouse line were assessed by CAT ELISA. In three independent transgenic mouse lines (TgM16, TgM27 and TgM37), CAT expression was observed in neuronal tissues such as cerebral cortex, cerebellum, olfactory bulb, hippocampus and trigeminal ganglia (Table 1). Especially high CAT expression was observed in trigeminal ganglia. In contrast, CAT expression was hardly detected in non-neuronal tissues (Table 1). A very small amount of CAT was detected in testis of TgM27 (Table 1). Western blot analysis of tissue extracts of TgM16 with polyclonal antibodies to CAT identified a protein band (approximately 24 kDa) in the trigeminal ganglia extract (Fig. 2A), which was consistent with the results of CAT ELISA analysis (Table 1). No protein with the same size was detected in other tissue extracts, although low levels of CAT were detected in CAT ELISA analysis. To analyse expression levels of CAT mRNAs in various tissues of TgM16, an RT-PCR analysis was performed. The expected PCR product was detected in trigeminal ganglia (Fig. 2B). In addition, weaker expression was observed in cerebellum. No PCR product was detected in other tissues. These results demonstrate that transgene expression was also strong in the trigeminal ganglia at the transcription level.

### Effect of reactivation stimuli on LAP activity

To investigate effects of putative reactivation stimuli on LAP activity, expression levels of the CAT gene under stress conditions were analysed by CAT ELISA. It has been reported that latently infected PRV in the trigeminal ganglia of mice can be reactivated by explantation of the ganglia (Osorio & Rock, 1992) or dexamethasone treatment.
The PRV LAP directs neuron-specific expression
Table 2. Effects of reactivation stimuli on CAT expression in the trigeminal ganglia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAT expression (pg mg⁻¹)</th>
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<tbody>
<tr>
<td>Explantation</td>
<td>48.2 ± 6.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>78.3 ± 9.4</td>
</tr>
</tbody>
</table>

(Tanaka & Mannen, 2002). On the basis of these findings, we tested these putative reactivation stimuli in the present study. As shown in Table 2, altered expression was not observed in the transgenic trigeminal ganglia at 24 h after the treatments.

**DISCUSSION**

Neuron-specific promoter activity of a 1015 bp Mlu–BamHI fragment (LAP; LAP 1 and LAP 2) was demonstrated in transgenic mice. Three transgenic mouse lines, in the absence of the viral proteins, displayed strong expression of the transgene in trigeminal ganglia. In addition, in situ hybridization demonstrated neuron-specific CAT expression in trigeminal ganglia. These data provide evidence that the PRV LAP directs neuron-specific expression in vivo. Only a small amount of CAT was detected in testis of TgM27. The absence of CAT expression in non-neuronal tissues of the other transgenic mouse lines may suggest that the expression in testis was an anomaly. Based on the available data, the HSV-2 LAT promoter drives abundant expression of LAT in trigeminal ganglia of transgenic mice (Wang et al., 2001). Taken together, these findings suggest that alphaherpesvirus LAT promoters may display strong promoter activity in trigeminal ganglia.

It is unclear why TgM16, which carries the highest transgene copy number, has a more restricted pattern of transgene expression than the lower copy transgenic lines. Some variability in the level of expression between different lines of transgenic mice as was seen in these mice is common (Hammer et al., 1987; Koedood et al., 1995; Mitchell, 1995). The fact that all three of the lines share the key expression patterns which are the focus of this paper is taken as evidence that the expression pattern is the result of specific activation of the LAP by host cell proteins. It is possible that the chromatin structure or sequence elements within the chromosomal DNA adjacent to the transgene can influence this expression. The copy number of the inserted transgene is another variable which has been suggested as a possible cause of differing levels of expression in transgenic lines; however, in many cases, the expression level is unrelated to the copy number of the transgene (Hammer et al., 1987; Koedood et al., 1995; Mitchell, 1995).

Many studies on the PRV LAT promoter using cultured cells have been performed with plasmids or recombinant viruses (Huang et al., 1994; Cheung & Smith, 1999; Jin & Scherba, 1999; Taharaguchi et al., 2002). However, it is likely that transcriptional regulation of the LAT promoter in cultured cells is different from regulation in vivo. Transgenic experiments were intended to examine regulation of the LAP in the more relevant in vivo context. The PRV LAP has been shown to be significantly active in both neuronal and non-neuronal cells (Cheung & Smith, 1999; Taharaguchi et al., 2002), suggesting that the LAP may be a pan-specific promoter. However, we demonstrated in the present study that the LAP is a neuron-specific promoter. Our results indicate that the in vivo tissue-specificity of the LAP correlates well with the known target of PRV latent infection.

Several lines of evidence suggest that LAT plays some role in facilitating reactivation of HSV from latency (Leib et al., 1989; Hill et al., 1990; Block et al., 1993; Bloom et al., 1994; Perng et al., 1994; Krause et al., 1995). Effects of putative reactivation stimuli on LAP activity were examined in the present study. However, explanation of the trigeminal ganglia and dexamethasone treatment did not affect the CAT expression levels. There are a number of possible explanations. The PRV LAP may not be the initial viral target for reactivation. It is possible that the trigeminal ganglion neurons which express the LAP-CAT transgene are different from the cells which maintain the latent viral genome. It is also possible that the latent viral genome DNA is regulated in a different way from the transgene which is within the host chromosome. The chromosomally located transgene may not be regulated precisely as the LAT promoter is in the context of the latent viral genome. Thus, the situation may indicate the limitations of the transgenic approach for studies on the regulatory mechanisms for LAT gene expression.

In the present study, it was demonstrated that expression levels of the transgene varied among neurons. These results may suggest that the level of activation of the PRV LAP can be altered by changes in the neuronal environment without any contribution from viral regulatory molecules. It is known that the transcription factors in a specific subset of neurons can be altered by changes in the neuron such as aging or differentiation (Herdegen & Leah, 1998). In fact, the HSV-1 ICP0 promoter in a specific subset of neurons was differentially regulated depending upon changes in the neuronal environment (Loiacono et al., 2002).

**Fig. 3.** CAT gene expression in neuronal tissues in TgM16. In situ hybridization was carried out using an antisense RNA probe (A, B, D, E, F). A sense probe was used as a negative control (C, G, H, I). (A–C) Trigeminal ganglion; (D, G) cerebral cortex; (E, H) cerebellum; (F, I) hippocampus. The sections were photographed without counterstaining. Scale bars, 100 μm.
The potential for neuronal regulation of PRV LAP in the absence of viral protein was shown in the present study. However, tissue-specific regulatory molecules which alter viral gene expression in neurons have not been identified. Further investigation of those regulatory molecules should be performed to elucidate fully PRV LAT gene expression. The specific expression of the PRV LAP-CAT transgene means that it is now possible to target expression of other heterologous genes to trigeminal ganglia.

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