Journal of NeuroVirology, 10: 116–122, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490279771

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Short Communication

The herpes simplex virus type 1 early gene (thymidine kinase) promoter is activated in neurons of brain, but not trigeminal ganglia, of transgenic mice in the absence of viral proteins

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Latent infection of sensory neurons and reactivation are necessary for maintenance of herpes simplex virus type 1 (HSV-1) in its host population. It has been proposed that the HSV-1 early gene, thymidine kinase (TK), may play an important regulatory role in this process. The authors used reporter transgenic mice to test whether sensory ganglia neurons could activate the HSV-1 TK reporter transgene in the absence of viral proteins. The reporter transgene was activated in subsets of neurons in the brain but was not activated in sensory ganglia neurons following a variety of experimental manipulations. These results do not support a role for TK in regulation of the latent viral genome. *Journal of NeuroVirology* (2004) **10,** 116–122.

Keywords: early; genes; HSV-1; human; transgenic mice; virus latency

Herpes simplex virus type 1 (HSV-1) can cause significant human diseases, including keratitis, conjunctivitis, encephalitis, and disseminated infections of the newborn (Whitley, 2001). Following the initial lytic infection of epithelium, the virus travels along axons to sensory ganglia neurons where it can cause either lytic or latent infection (Stevens, 1975, 1989). Latent infection of sensory ganglia neurons is the key to maintenance of HSV-1 in the human population. Periodic reactivation results in spread of the virus to new hosts. The mechanism that controls HSV-1 latency and reactivation in neurons is not understood. Transcription in the latent HSV-1 genome is limited to the production of latency-associated transcripts (LATs) (Croen *et al*, 1987; Deatly *et al*, 1987; Mitchell *et al*, 1990; Rock *et al*, 1987; Stevens *et al*, 1987). Evidence in rabbit models suggests that LATs may play a role in reactivation (Hill *et al*, 1990; Perng *et al*, 1996).

Most hypotheses that attempt to explain the regulation of the latent HSV-1 genome include the idea that neuronal transcriptional proteins regulate one or more viral promoters in the latent genome. According to this idea, reactivation would result from activation of one or more viral promoters by neuronal transcriptional proteins. It has been suggested that viral immediate-early genes are the targets of these neuronal transcriptional regulatory proteins and thus may control latent and reactivated infections of HSV-1 (Fraser and Valyi-Nagy, 1993; Garcia-Blanco and Cullen, 1991; Tensor *et al*, 1993). Previous experiments have demonstrated that the viral ICP0 promoter is activated in sensory ganglia neurons of transgenic mice at different stages of development and following injury (Loiacono *et al*, 2002, 2003). It has also been suggested that the viral early gene (thymidine kinase, TK) may be an important target of neuronal transcriptional proteins and that it may be important in regulation of latency and reactivation in sensory neurons (Tal-Singer *et al*, 1997). In relation to this hypothesis of latent HSV-1 regulation, it is important to determine whether *in vivo* sensory ganglia neurons have the capacity to differentially regulate the expression of the viral early gene (TK) in the absence of viral proteins.

In order to study the ability of neurons to activate the HSV-1 TK gene, reporter transgenic mice that

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The authors thank Lawrence Butcher, Jeff Croarkin, Jim Burrow, and Cherie Chapman for technical assistance. CML was supported by a NIH postdoctoral fellowship award (EY07007). This work was supported by NIH grants EY11833 and AI01552 to WJM.

Received 27 May 2003; revised 7 October 2003; accepted 13 October 2003.

contain the TK promoter fused to the *Escherichia coli* β-galactosidase coding sequence were generated and analyzed. Three founder lines containing the TK–β-galactosidase reporter transgene were generated and studied in detail: TgN(HSVTKRp)1wm (Tg6748), TgN(HSVTKRp)2wm (Tg6751), and TgN(HSVTKRp)3wm (Tg6752). The transgenic lines were screened for expression of the reporter transgene in the presence and absence of HSV-1. Viral infection resulted in β-galactosidase–positive cells in the trigeminal ganglia of four out of four mice from each of the three lines (Tg6748, Tg6751, and Tg6752). No β-galactosidase–labeled cells were detected in trigeminal ganglia of age-matched mock-inoculated TK–β-galactosidase transgenic mice (Tg6748, Tg6751, and Tg6752). No β-galactosidase–labeled cells were detected in trigeminal ganglia of nontransgenic littermates infected with HSV-1 (F). This experiment confirmed that the transgene (TK–β-galactosidase) was appropriately expressed in the presence of viral proteins in sensory ganglia neurons.

TK reporter transgenes were expressed in anatomically distinct subsets of brain neurons in uninfected adult mice in the absence of viral proteins. TK–β-galactosidase transgenic mice from each of three lines (Tg6748, Tg6751, and Tg6752) contained low to moderate numbers of β-galactosidase–positive cells in the brains of uninfected adult mice (Table 1, Figure 1). β-Galactosidase–positive neurons were present in the following anatomically defined regions of the brain in TK reporter transgenic mice: cerebral cortex, cerebellum, caudate putamen, hippocampus, thalamus, geniculate nucleus, cingulum, and superior colliculus (Table 1; Figure 1A–D). Small numbers of positive neurons were present in reticular nucleus and vestibular nucleus (Table 1). The β-galactosidase–positive cells were determined to be neurons by morphological criteria. No β-galactosidase–positive cells were detected in the white matter of brains from any of the transgenic lines (Tg6748, Tg6751, and Tg6752) None of five uninfected adult mice from each TK reporter transgenic line (Tg6748, Tg6751, and Tg6752) contained β-galactosidase–positive cells in trigeminal ganglia or spinal cord. All non-neural tissues of TK–β-galactosidase transgenic mice were negative for β-galactosidase labeling. Nontransgenic littermates from the above described transgenic lines contained no positive cells in neural or non-neural tissues (Table 1).

Table 1 Distribution of β-galactosidase labeling in mice containing the HSV-1 TK reporter transgene

	TK			*Non-Tg controls*
	Tg6748	*Tg6751*	*Tg6752*	
Cerebellum (Purkinje cell layer)	1	3	5	0
Vestibular nucleus	1	1	0	0
Reticular nucleus	2	2	0	0
Cerebral cortex	5	5	5	0
Cingulum	0	5	4	0
Caudate putamen	5	4	5	0
Hippocampus	2	5	4	0
Thalamus	5	3	5	0
Geniculate nucleus	2	3	2	0
Superior colliculus	2	4	3	0
Trigeminal ganglia	0	0	0	0
Spinal cord	0	0	0	0

Note. Each number represents the number of mice out of five that contained β-galactosidase–labeled cells in the indicated anatomic regions. None out of five mice from each transgenic line contained any β-galactosidase–labeled cells in cornea, adrenal, kidney, liver, spleen, heart, lung, or intestine.

β-Galactosidase–positive cells in brain were identified as neurons in colabeling experiments. β-Galactosidase–positive cells in the cerebral cortex of TK–β-galactosidase (Tg6751) transgenic mice were colabeled for the neuron-specific marker, neurofilament protein (Figure 1E). Neurofilament staining (brown) was seen in the neuronal cytoplasm whereas β-galactosidase label (blue) was located in the nucleus (Figure 1E). Subsets of neurons that contained β-galactosidase as well as subsets of neurons that did not contain β-galactosidase were labeled for neurofilament protein. Control mouse antibody was used on adjacent sections of cerebral cortex and no specific labeling was detected (Figure 1F).

TK reporter transgenes were not expressed in neurons of the trigeminal ganglia in uninfected newborn and adult mice. Trigeminal ganglia of newborn and adult (8-week-old) mice from each of the three lines of TK reporter transgenic mice were analyzed. No β-galactosidase–positive cells were detected histologically in the trigeminal ganglia of newborn or adult (8-week-old) mice from any of the transgenic lines (Tg6748, Tg6751, and Tg6752) (Table 2). No β-galactosidase–positive cells were present in the trigeminal ganglia of nontransgenic newborn and adult control mice.

Ultraviolet (UV) irradiation of corneas and sensory nerve endings failed to activate the HSV-1 TK promoter in trigeminal ganglia of transgenic mice in the absence of viral proteins. Experiments were performed to determine whether a stimulus such as UV irradiation, which results in reactivation of latent HSV-1 from sensory neurons, can cause activation of the viral TK promoter. HSV-1 TK reporter transgenic mice (Tg6748, Tg6751, and Tg6752) were exposed to UV light as described in Materials and Methods. The β-galactosidase levels in trigeminal ganglia of exposed mice were compared with β-galactosidase levels in trigeminal ganglia of transgenic mice of the same genotype that were not exposed to UV light. No significant increase in the TK promoter activity as evidenced by the measurement of β-galactosidase levels was detected in trigeminal ganglia of the UV-treated mice (Table 2).

In summary, anatomically distinct subsets of neurons in the brain were able to activate the HSV-1 TK promoter in the absence of viral proteins in TK

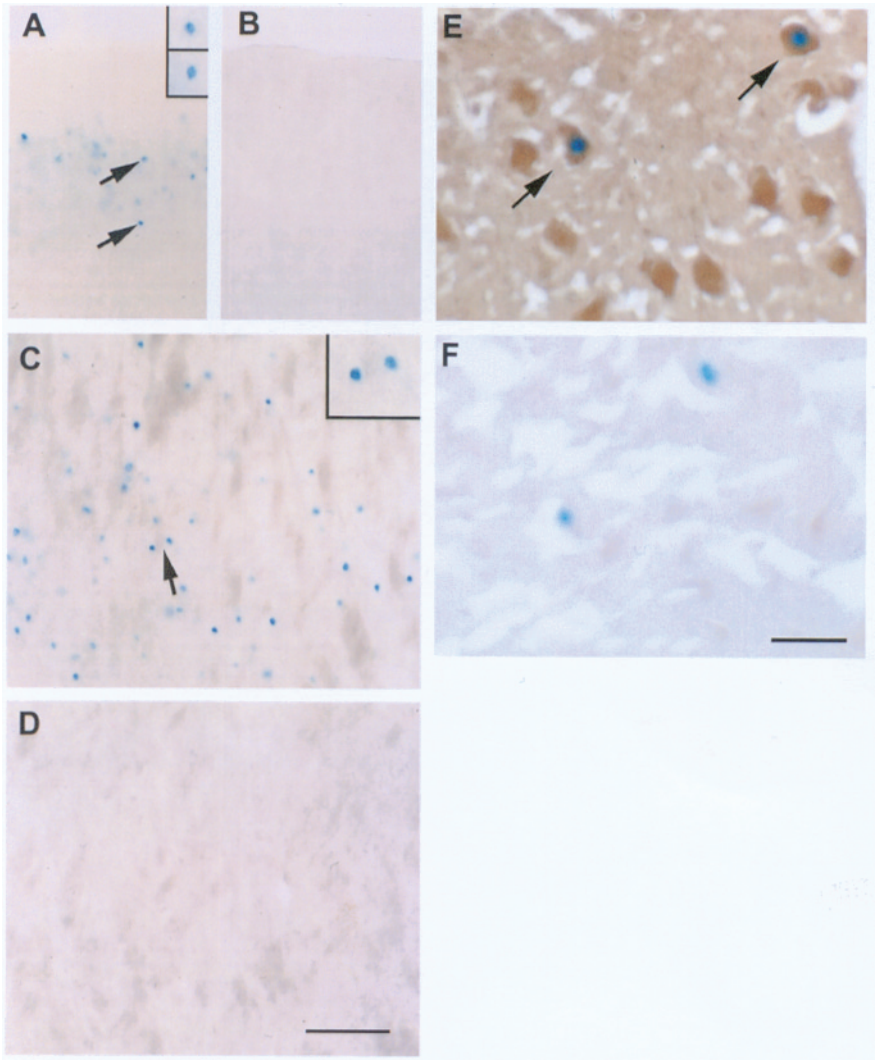

Figure 1 Expression of β-galactosidase in neurons of the cerebral cortex and thalamus in mice containing the HSV-1 TK reporter transgene in the absence of viral proteins. (**A**) Coronal section through the cerebrum of a TK reporter transgenic mouse (Tg6748). The inset is a higher magnification of β-galactosidase–labeled neurons indicated by the arrows. (**B**) Coronal section through the cerebrum of a nontransgenic control mouse showing no labeled cells. (**C**) Coronal section through the thalamus of a TK reporter transgenic mouse (Tg6748). The inset is a higher magnification of β-galactosidase–labeled neurons indicated by the arrow. (**D**) Coronal section through the thalmus of a nontransgenic control mouse showing no labeled cells. (**E**) Section of cerebrum of a TK reporter transgenic mouse (Tg6748) containing neurons doubly labeled for β-galactosidase and the neuronal marker, neurofilament. The brown neurofilament staining is present in cytoplasm surrounding the β-galactosidase–positive neuronal nuclei. Both neurons that express β-galactosidase and neurons that lack β-galactosidase expression are labeled for neurofilament. (**F**) Section of cerebrum containing β-galactosidase–labeled cells that have been reacted with monoclonal anti-bovine CD3 in the immunocytochemical assay as a negative control. Bar is equal to 60 μm in **A–D**, 25 μm in **E–F**.

reporter transgenic mice. These results show that it is possible for neuronal transcriptional proteins in some subsets of neurons to activate the TK promoter in the absence of viral proteins. It is of interest to compare the distribution of neurons that activated the HSV-1 early (TK) promoter with those that activated the immediate-early (ICP0, ICP27, and ICP4) and late (gC) promoters in transgenic mice reported in earlier studies (Loiacono *et al*, 2002, 2003; Mitchell, 1995). TK–β-galactosidase reporter transgenic mice had positively labeled neurons in the following anatomically defined regions of the nervous system: cerebral cortex, hippocampus, thalamus, caudate putamen, cingulum, vestibular nucleus, cerebellum, reticular nucleus, geniculate nucleus, and superior colliculus. TK reporter transgenic mice did not contain any positive neurons in other regions of the nervous system, including amygdala, trigeminal ganglia, or spinal cord. ICP0-reporter transgenic mice contained positive neurons in the cerebral cortex, hippocampus, thalamus, cingulum, vestibular nucleus, cerebellum, amygdala, lateral septal nucleus, superior colliculus, spinal cord, and trigeminal ganglia. ICP27 reporter transgenic mice contained positive neurons

Table 2 β-Galactosidase expression in trigeminal ganglia of TK reporter transgenic mice

Transgenic line	*Newborn none*	*Adult none*	*Adult HSV-1*	*Adult UV*
Tg6748	0/5[a]	0/5	4/4	0/20[b]
Tg6751	0/5	0/5	4/4	0/20
Tg6752	0/5	0/5	4/4	0/20

Note. Mock-infected adult mice (5 from each transgenic line) and adult mice not exposed to ultraviolet light (20 from each transgenic line) were negative. None refers to no manipulation of the mice examined. HSV-1 refers to animals that were infected with 1×10^7 PFU of HSV-1 via the cornea. UV refers to animals that were exposed to UV irradiation. Adults are 8 to 10 weeks old and newborns are 1 day old.
[a]The numerator in each of the first three columns represents the number of mice that contained β-galactosidase–positive neurons in trigeminal ganglia. The denominator represents the total number of mice examined.
[b]The numerator in this column represents the number of mice that contained β-galactosidase activity above background in homogenates of trigeminal ganglia. The denominator represents the number of mice tested.

in the cerebral cortex, hippocampus, thalamus, cingulum, vestibular nucleus, amygdala, lateral septal nucleus, and trigeminal ganglia. ICP4 reporter transgenic mice had a distribution of positively labeled neurons, which included the following: cerebral cortex, hippocampus, cerebellum, mammillary nucleus, induseum griseum, superior colliculus, spinal cord, and trigeminal ganglia. Late gene (gC) reporter transgenic mice contained no labeled neurons. Although there are a number of differences in the distribution of positively labeled neurons between the different reporter transgenes in the brain, the most notable difference is in trigeminal ganglia. The immediate-early reporter transgenes are expressed in sensory neurons under differential conditions, whereas no expression of the early gene (TK) reporter transgene was detected in trigeminal ganglia under any of the previously described conditions. The HSV-1 TK reporter was not activated in sensory neurons of newborn transgenic mice, adult transgenic mice, or adult transgenic mice that had undergone UV treatment of nerve endings in the cornea. This is in contrast to previous experiments that showed that the HSV-1 immediate-early genes (ICP0 and ICP4) were differentially regulated in sensory ganglia neurons of mice of different ages and that the ICP0 promoter was activated in sensory neurons following UV treatment. Thus transcription factors in sensory neurons of trigeminal ganglia do not activate the TK promoter to detectable levels in the absence of viral proteins and they apparently do not activate the TK promoter following a reactivation stimulus.

The major drawback of these experiments is that the viral TK promoter cannot be examined in its natural context in the viral genome in the correct position of the gene. However, it is most important to first determine the potential for neuronal regulation of the HSV-1 TK promoter in the absence of viral proteins. Most experiments that have examined the activity of viral promoters in cultured cells have been performed with the viral promoter inserted into a plasmid. These studies have yielded valuable data about the regulation of viral genes, including the HSV-1 TK gene. It should also be noted that one study has suggested that the latent HSV-1 genome is maintained in a state that is similar to chromosomal DNA (Deshmane and Fraser, 1989). Other studies have used reporter transgenes to study the function of viral promoters in transgenic mice (Aiba-Masago *et al*, 1999; Baskar *et al*, 1996; Fritschy *et al*, 1996; Koedad *et al*, 1995; Loiacono *et al*, 2002, 2003; Mitchell, 1995; van den Pol and Ghosh, 1998). The focus of these experiments was to compare the level of activation of the TK–β-galactosidase reporter transgene with the results of earlier studies on immediate-early promoters using the same reporter enzyme assay. Measurement of the β-galactosidase enzyme may not reflect the precise kinetics of promoter activity (Margolis *et al*, 1993); however, this should not impact the results of these experiments.

Different neuronal factors are apparently required for activation of each of the immediate-early viral promoters (ICP0, ICP4, and ICP27) and early promoter (TK) in transgenic mice in the absence of viral proteins. The TK promoter is unlikely to be a target for transcriptional activation of the latent HSV-1 genome in trigeminal ganglia neurons.

Materials and methods

Generation and identification of transgenic mice
The promoter regulatory region of the thymidine kinase gene of HSV-1 was fused to the bacterial β-galactosidase coding sequence using standard techniques (Sambrook *et al*, 1989) to produce a reporter transgene (Figure 2). The promoter regulatory region (nucleotides −197 through +32 with respect to the transcription start site of TK) (McKnight *et al*, 1981) was removed from the pRL-TK plasmid containing the HSV-1 TK promoter. The resulting PvuII-HindIII fragment was made blunt ended and ligated in the proper orientation into the pNlacF plasmid. The pNlacF plasmid contains the *E. coli* β-galactosidase coding sequence and a simian virus 40 nuclear translocation signal (Mercer *et al*, 1991). The XbaI-HindIII fragment containing the viral

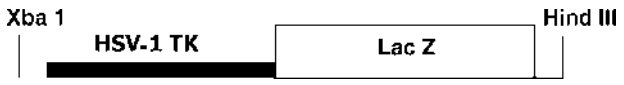

Figure 2 Diagram of the HSV-1 TK reporter transgene. The TK promoter DNA fragment included nucleotides −197 through +32 of the TK gene. This DNA fragment was fused to the coding seqence for the β-galactosidase gene of *E. coli*, which also contained a nuclear translocation signal from simian virus 40.

promoter fused to β-galactosidase from the final reporter transgene construct was isolated and purified as previously described (Mitchell, 1995). Approximately 200 copies of the construct were injected (Hogan *et al*, 1994; Mitchell *et al*, 1993) into (C57BL/6xC3H) × (C57BL/6xC3H) one-cell embryos. Three lines of TK reporter transgenic mice were generated and characterized. Transgenic lines were established from founders through brother-sister matings. For each line, heterozygous transgenic mice and their nontransgenic littermates were used in experiments. Mice were identified as transgenic or nontransgenic by polymerase chain reaction (PCR) of tail DNA (Mitchell *et al*, 1993) for the β-galactosidase sequence (Mitchell, 1995). The primers for the β-galactosidase coding sequence were GCATCGAGCTGGGTAATAAGCGTTGGCAAT and GACACCAGACCAACTGGTAATGGTAGCGAC.

Analysis of transgene expression in uninfected mice
Adult mice, 8 to 10 weeks old, from each of the three transgenic lines were euthanized and tissues were removed and stored at −70°C. The following tissues were examined for each of five mice from each transgenic line: brain, trigeminal ganglia, spinal cord, cornea, heart, lung, liver, spleen, kidney, adrenal gland, and small intestine. Whole trigeminal ganglia, cornea, and brain (every other section of the brain) were examined for each animal. Four representative sections of all other tissues for each animal were studied. Trigeminal ganglia and eyes were fixed as whole tissues in 4% paraformaldehyde for 30 min immediately following removal from −70°C. All other tissues were sectioned at 40 μm with a cryotome, adhered to glass slides, and fixed in 4% paraformaldehyde for 30 min. Tissue sections, whole eyes, and whole ganglia were washed in phosphate-buffered saline (PBS) for 5 min and incubated in substrate solution (Mitchell, 1995) for 14 to 18 h at 37°C. The substrate solution contained 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) per ml, 120 μl of 10% Nonidet P-40, and 100 μl of 1% sodium deoxycholate per 20 ml. Labeled tissue sections and whole tissues (eyes and ganglia) were washed for 5 min in PBS. Cryotome sections, trigeminal ganglia (thinly sliced with a razor blade), and corneas (removed from eyes) were mounted on glass slides and coverslips were sealed with permount. Neuroanatomical locations were assigned according to the *Mouse Brain Atlas*: C57BL/J Coronal found at the *Mouse Brain Library* website (http://www.nervenet.org/mbl/).

Analysis of transgene expression in virus infected mice
Four mice from each of the three TK reporter transgenic lines (Tg6748, Tg6751, and Tg6752) and four nontransgenic control mice were inoculated with 1×10^7 plaque-forming units (PFU) of HSV-1 strain F per eye following anesthesia and corneal scarification (Chang *et al*, 2000; Maggs *et al*, 1998; Mitchell *et al*, 1994). Four mice from each line were mock inoculated using medium containing no virus. Mice were euthanized 4 days following inoculation and trigeminal ganglia were analyzed as described above for the presence of β-galactosidase–labeled cells.

Analysis of transgene expression in trigeminal ganglion neurons of newborn and adult mice
In order to determine whether changes in neuronal differentiation and neuronal transcriptional proteins might influence activation of the TK promoter, trigeminal ganglia of newborn and adult (8-week-old) mice from each of the three lines of TK reporter transgenic mice were analyzed. β-Galactosidase assays were performed as described previously and both ganglia from each mouse were examined for positively labeled neurons.

Reactivation model
Uninfected mice were anesthetized and exposed to an UV transilluminator (302 nm wavelength) for 4 min per eye. Two days following UV exposure, trigeminal ganglia were dissected and removed from each mouse. The ganglia were homogenized and total protein content of each sample was measured using the Bio-Rad protein assay. One hundred micrograms of each sample was assayed in a total of 200 μl of lysis buffer containing 8 mM chlorophenol red β-D-galactophranoside (CPRG). Samples were incubated for 2.5 h and each sample was read at 595 nm on a Beckman DU 7400 spectrophotometer. β-Galactosidase activity was determined for each sample as the percentage of the spectrophotometer reading above the negative control.

Colabeling of β-galactosidase–positive cells for a neuron-specific marker in brain of TK reporter transgenic mice
Five-millimeter coronal slices of brain were fixed in 4% paraformaldehyde for 30 min, washed in PBS for 5 min, and incubated in substrate solution containing X-gal (as described above) for 18 to 20 h. Samples containing β-galactosidase–positive cells were embedded in paraffin and sectioned at 6 μm. Sections of brain were mounted on positively charged glass slides and deparaffinized using clear-rite 3. Immunohistochemical localization of the mid-range molecular weight neurofilament protein (Caccarno *et al*, 1989; Zindy *et al*, 1999) was carried out with a standard biotin-avidin peroxidase assay as described previously (Maggs *et al*, 1998; Mitchell, 1995; Mitchell *et al*, 1993, 1994). The primary antibody (used at a 1:40 dilution) was a mouse monoclonal antibody directed against neurofilament protein (molecular weight 160,000 Da) (Sigma). As a control, adjacent sections were incubated with mouse anti-bovine CD3 monoclonal antibody (1:40 dilution).

The antibody-biotin-avidin–horseradish peroxidase complexes were visualized by incubation of sections in diaminobenzidine (DAB). Endogenous peroxidase activity was blocked by a 30-min incubation in methanol containing 3% hydrogen peroxide. Sections were washed in PBS and incubated in a serum-free protein blocker (Dako Corporation). The M.O.M. immunodetection kit (Vector Laboratories) was used according to manufacturer's instructions to reduce the background staining associated with using mouse antibody on mouse tissue. The reaction was terminated by washing the slides in distilled water. The slides were dehydrated and coverslips were sealed with permount.

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