Neuron-Specific Activation of Murine Cytomegalovirus Early Gene e1 Promoter in Transgenic Mice

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The brain is the main target in congenital cytomegalovirus (CMV) infection and immunocompromised patients. No definite evidence that a CMV has special affinity for the central nervous system (CNS) has been published. Here, we generated transgenic mice with an e1 promoter/enhancer region connected to the reporter gene lacZ. Surprisingly, expression of the transgene was completely restricted to the CNS in all lines of transgenic mice. The transgene was expressed in subpopulation of neurons in the cerebral cortex, hippocampus, diencephalon, brainstem, cerebellum, and spinal cord in all of the lines. Non-neuronal cells in the CNS were negative for transgene expression. Activation of the transgene was first observed in neurons of mesencephalon in late gestation, and then the number of positive neurons increased in various parts of the brain as development proceeded. Upon infection of the transgenic mouse brains with MCMV, the location of the activated neurons became more extensive, and the number of such neurons increased. These results suggest that there are host factor(s) that directly activate the MCMV early gene promoter in neurons. This neuron-specific activation may be associated with persistent infection in the brain and may be responsible for the neuronal dysfunction and neuronal cell loss caused by CMV infection. (*Am J Pathol* 2003, 163:643–652)

Human cytomegalovirus (HCMV) is the most significant infectious cause of severe neuronal disorders of the central nervous system (CNS) due to intrauterine infection in humans.¹ It is estimated that approximately 5 to 10% of infected infants have generalized inclusion disease at birth, with symptoms such as microcephaly, periventricular calcification, and microphthalmia.^{2–4} Brain disorders such as mental retardation, sensorineural hearing loss,

visual disorder, seizures, and epilepsy have also been reported after subclinical congenital HCMV infection.^{5,6} In healthy individuals, infection with HCMV is usually asymptomatic, but the virus can cause severe or fatal disease in immunocompromised patients.⁷ HCMV has become the most frequent opportunistic brain infection in acquired immunodeficiency syndrome (AIDS), resulting in HCMV encephalitis/encephalopathy.^{8–10} Although the brain is the main target for HCMV infection, the pathogenesis of the brain disorders after long-term infection has not been elucidated because of the limitations in studying human subjects.

Murine cytomegalovirus (MCMV) has been used as a model of human CMV infection with respect to pathogenesis, tissue tropism, persistent infection, and latency.^{11–13} Similarly to the gene of HCMV and other herpesviruses, MCMV genes are expressed in three sequential phases: immediate-early (IE), early, and late.¹⁴ In brains infected with CMV, infected cells consist preferentially of glial cells in the periventricular regions in both HCMV infection^{2,15} and in mouse models.^{16,17} These infected cells underwent lytic infection due to strong expression of the IE antigen and late antigen as the viral DNA increased.¹⁶ In accordance with these results, the MCMV IE gene-promoter directed glial-specific expression in the transgenic mice.^{18,19}

In chronic infection, the early nuclear antigen tends to be retained in neurons for a prolonged time after infection.²⁰ Previously we made a monoclonal antibody specific for the viral early nuclear antigen,²¹ which is the product of MCMV early gene e1 (M112–113),²² corresponding to HCMV early gene (UL112–113) from which nuclear DNA-binding phosphoprotein is expressed.²³ The neuron-specific expression of the early gene in the chronic infection of the developing mouse brain may be associated with the pathogenesis of brain disorders such as neuronal cell loss or neuronal dysfunction after long-term infection with CMV in the brain.

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In the present study, we showed that there is neuron-specific activation of the enhancer/promoter of the MCMV early e1 gene in transgenic mice. This activation can be enhanced by infection with MCMV in the brain. This model may be useful for elucidating mechanisms of the neuron-specific gene expression and for studying the pathogenesis of persistent CMV infection and neuronal dysfunction of the brain.

Materials and Methods

Construction and Preparation of Transgene

The e1 gene region, including the coding region and the promoter sequence, of MCMV (Smith strain) was reported to be restricted to a region between map units 0.709 and 0.721 of the genome in the *HindIII* F fragment.²⁴ A plasmid containing the F fragment from the MCMV *HindIII* library was selected using polymerase chain reaction (PCR) with primers (5' primer, 5'-CGGGGTACCCGAGGAGCGC-CACTAGGTTG-3', 3' primer, 5'-ATAGAAGCTTTGGTCT-GCTAAATGCGAAGATCG-3') specific for the e1-promoter region. Both primer sequences were obtained from the GenBank database (Accession No. L07320) using Primer3 software. After selection of the F-fragment-containing plasmid, the MCMV-e1-promoter DNA fragment (1572 bp; -1534 to +38) was excised and blunt-ended by treatment with Klenow enzyme (TaKaRa, Tokyo, Japan). Then the blunt-ended fragment was ligated into the *Sma*I-digested pnlacF plasmid. The pnlacF plasmid containing the nuclear localization signal from SV40, the *Escherichia coli* β -galactosidase (β -gal) coding sequence, and the poly(A) tract and an intron from the mouse protamine gene, was provided by Dr. R. D. Palmiter of the University of Washington.²⁵ The resulting plasmid was designated pMCMV-e1-pro-lacZ. The transgene fragment (5.2 kb) used for microinjection was isolated from the plasmid DNA digested with *KpnI* and *BglII*.

Generation of Transgenic Mice

Transgenic mice were produced by standard techniques.²⁶ Purified linearized DNA was injected into the pronuclei of fertilized ova derived from BDF1 (C57BL/6 female $\times$ DBA/2 male) mice. After injection, ova were transferred to oviducts of pseudopregnant female ICR mice. Transgenic founders were identified by PCR of genomic DNA prepared from tail tips using standard procedures. PCR was performed with MCMV-e1-pro-lacZ-specific primers as described above for 35 cycles of 1 minute at 94°, 1 minute at 60°, and 1 minute at 72°. The reaction products were electrophoresed on a 2.0% agarose gel that was then stained with ethidium bromide and photographed.

Southern Blot Analysis

Transgenic founders identified by PCR were further analyzed by Southern blotting. The genomic DNA and control DNA digested with *Bam*HI, which cuts twice in the lacZ

region of the transgene, were electrophoresed on 0.9% agarose gels and transferred onto nylon membranes (MSI, Westborough, MA). The membranes were then probed using a ³²P-labeled transgene *Bam*HI fragment and analyzed with Bio Imaging Analyzer (BAS1000Mac, FUJIX, Tokyo, Japan). Founder lines were established from transgenic mice that transmitted MCMV-e1-pro-lacZ to their progeny.

X-Gal Histochemical Staining

Expression analysis was performed on F1 or F2 mice obtained by mating hemizygous transgenic animals with outbred C57BL/6 mice. The mice were anesthetized with diethyl ether and perfused with 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PB). After perfusion, tissues were removed and fixed in freshly prepared 4% PFA in 0.1 mol/L PB for 20 minutes at room temperature. The organs and tissues were incubated in a reaction mixture containing 1 mg of 5-bromo-4-chloro-3-indolyl- β -galactosidase (X-Gal) per ml, 5 mmol/L K₄Fe(CN)₆, 5 mmol/L K₃Fe(CN)₆ in 0.1 mol/L PB (pH 7.3) overnight at room temperature.²⁵ After staining, each tissue was embedded in paraffin and sectioned.

LightCycler RT-PCR for Quantification of β -Galactosidase mRNA

RNA, which was extracted from each freshly removed transgenic mouse's tissue using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, was treated with RNase-free DNaseI (TaKaRa, Otsu, Japan) and reverse-transcribed into cDNA using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California). Each sample cDNA was amplified PCR using primers specific to the β -gal and mouse hypoxanthine guanine phosphoribosyl transferase (HPRT), those sequences are as follows (GenBank): β -gal, 5'-GCGAATACCTGTTCCGTCAT-3' and 5'-ACATCCAGAGGCACTTCACC-3' for a 97-bp fragment; mouse HPRT, 5'-TGCTCGAGATGTCATGAAGG-3' and 5'-TGTAATCCAGCAGGTCAGCA-3' for 98-bp fragment. LightCycler RT-PCR was performed as described by Schaade et al.²⁷ An aliquot of 2 μ l of the extracted cDNA was added to 18 μ l of reaction mixture containing 2 mmol/L MgCl₂, 0.5 μ mol/L concentration of each primer for the β -galactosidase and mouse HPRT and 2 μ l of LightCycler-FastStart DNA Master SYBER Green I (Roche Biochemicals, Mannheim, Germany), respectively. The amount of mRNAs was quantified by a LightCycler (Roche). The experimental PCR protocol was as follows: an initial 10 minutes at 95° for FirstStart TaqDNA polymerase activation, followed by 40 cycles of 15 seconds denaturation at 95°, 5 seconds annealing at 55°, and 10 seconds extension at 72°. Each melting temperature of the specific probes was as follows: β -gal, 83.6°; mouse HPRT, 84.1°, respectively.

Quantification of β -gal cDNA and mouse HPRT cDNA was performed with eight 10-fold serial dilutions of each pre-treated PCR products, those DNA concentrations

Figure 1. Structure of the transgene, and Southern blot analysis. **A:** Construct used to generate MCMV-e1-pro-lacZ transgenic lines (transgene; 5.2 kb). The MCMV e1 (M112–113) promoter (MCMV-e1-pro) (nucleotides – 1534 to + 38) was inserted into the pnlacF vector,²⁵ containing nuclear localization signal (N) and polyadenylation signals from the mouse protamine (mP1) gene. For the preparation of transgene, the vector was digested with *KpnI* and *BglII*. **B:** Southern blotting of the tail DNA from the four transgenic lines. Aliquots of 10 μ g of tail DNA were digested with *BamHI*, electrophoresed in a 0.9% agarose gel, transferred to a nylon membranes, and probed with a ³³P-labeled *BamHI* fragment (3.0 kb; **A**). Control lanes show increasing amounts: 0 copy (**lane 1**), 1 copy (**lane 2**), 5 copies (**lane 3**), 10 copies (**lane 4**), 20 copies (**lane 5**) of *BamHI*-digested mixtures of MCMV-e1-pro-lacZ with 10 μ g of normal mouse tail DNA. **Lane 6**, Tg-029; **lane 7**, Tg-041; **lane 8**, Tg-046; **lane 9**, Tg-033.

were measured by spectrophotometry at 260 nm and adjusted the copy numbers by PCR grade water dilution. The copy numbers of β -gal cDNA from organs were expressed as divided by those of mouse HPRT cDNA for normalization. The same samples were also subjected to ordinary PCR using the same primers as shown above, and the PCR products were electrophoresed on 2% agarose gel.

Immunohistochemical Analysis

For immunohistochemical analysis,¹⁶ mice from embryonic to adult stages were anesthetized and perfused with 4% PFA in 0.1 mol/L PB. Tissues were removed, fixed with 4% PFA in 0.1 mol/L PB again for 5 to 7 days, embedded in paraffin, and sectioned. For the developmental analysis, mice at embryonic day 18.5 (E18.5), postnatal day 3 (P3), P7, and P28 were processed in the same way. After deparaffinization and rehydration, sections were pretreated with 0.3% hydrogen peroxide to block endogenous peroxidase, and then with reagents to block endogenous biotin (Biotin Blocking System, Dako, USA). The sections were incubated with rabbit anti- β -galactosidase antibody (anti- β -gal; Cappel, Durham, NC) for 30 minutes at room temperature, followed by horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Dako, Tokyo, Japan), then colored with 3-amino-9-ethyl-carbazole (AEC; Dako, Tokyo, Japan), or incubated with biotin-conjugated goat anti-rabbit IgG (Nichirei, Tokyo, Japan), and alkaline phosphatase-conjugated streptavidin (Dako, Tokyo, Japan), and then colored with fast blue BB. For confirming the cell-type specificity of the transgene-expressing cells, fast blue BB-stained transgenic brain specimens were double-stained with mouse monoclonal antibody (mAb) specific for neuron-specific- α -enolase (NSE) (Novocastra Lab. Ltd., Newcastle, UK), β -tubulin isotype III (Sigma, St. Louis, MO), MAP2 (Sigma), or with rabbit antibody specific for glial fibrillary acidic protein (GFAP) (Dako, Tokyo, Japan). For detection of MCMV-infected cells, infected brains were immunohisto-

chemically stained using mouse mAb D5, specific for the MCMV early nuclear antigen,²¹ which is the product of e1 gene (M112–113).²²

Intracerebral MCMV Inoculation of Neonatal Mice

Virus preparation of MCMV Smith strain was described previously.²⁸ The titer of the purified virus stock was 4×10^8 plaque forming units (PFU)/ml. The intracerebral infection of neonatal mice with MCMV was done within 24 hours of their birth.²⁹ The neonatal mice were outbred from individual transgenic founder mice, and half of the total neonates from a pregnant transgenic founder were inoculated with MCMV, while the other half were injected with Minimum Essential Medium (MEM) as a negative control. The viral stock was diluted with MEM to an appropriate titer (10^5 PFU/ μ l), and 10^5 PFU of virus was injected into the right side of the cerebral hemisphere through the cranial bone using a microsyringe. The negative inoculation control group was injected with the same volume of MEM in the same way. Positive cells were counted in the cerebral cortex of the coronal sections and the numbers were expressed per cortex area or per hippocampal area of each cerebral hemisphere. The data of three animals in each transgenic line were averaged.

Results

Generation of MCMV e1-pro-lacZ Transgenic Mice

Transgenic mice that expressed the lacZ gene under the transcriptional control of the MCMV-e1-promoter (nucleotides – 1534 to + 38; Figure 1A) were generated for analysis of the tissue-specific regulation of this promoter. Among the founder mice with transgene-integration detected by PCR, four independent transgenic lines were

Figure 2. β -galactosidase expression in the CNS in 4-week-old transgenic lines. The sliced brains and spinal cords from three transgenic lines (Tg-029, Tg-041, Tg-046, and Tg-033) were stained for X-Gal. **A:** Coronal cerebral slices through frontal lobe. **B:** Coronal cerebral slices through the mammillary bodies. **C:** Sagittal slices of the brainstem and cerebellum. **D:** Spinal cords. In Tg-033, expression of the transgene was not detected in normal conditions.

found to express the transgene by X-Gal staining. Southern blotting analysis of the four transgenic lines is shown in Figure 1B. The *Bam*HI fragment (3.0 kb) from MCMV-e1-pro-lacZ was detected in these transgenic lines after digestion with *Bam*HI (Figure 1B, lanes 6 to 9). The copy numbers of the transgene in the transgenic lines were determined by using NIH image software. The lines contained various transgene copy numbers: 10 copies in Tg-029 (lane 6), 32 copies in Tg-041 (lane 7), 3 copies in Tg-046 (lane 8), and 12 copies in Tg-033 (lane 9). The male founders were crossed with non-transgenic C57BL/6 mice, and transmitted the gene to ~ 50% of the offspring. These hemizygous males were mated with female C57BL/6 mice, and the offspring were used for further experiments.

Expression of MCMV-e1-pro-lacZ in Transgenic Mice

Among four lines of founder mice bearing the MCMV-e1-pro-lacZ transgene, three lines (Tg-029, Tg-041, and Tg-046) were found to express the transgene as indicated by X-Gal staining of 4-week-old transgenic mice. In one line (Tg-033), expression of the transgene was not detected in normal conditions. However, the expression was detected by induction with MCMV infection (shown below). In all of the transgenic lines, expression of the transgene was restricted to the CNS (Figure 2). X-gal-positive-regions were seen in the cerebral cortex, hippocampus, diencephalon, cerebellar cortex, brainstem, and ventral dorsal horn of the spinal cord (Figure 2). Expression of β -gal was also assayed quantitatively by the LightCycler

Figure 3. Expression of β -galactosidase of the organs from Tg-029 assayed quantitatively by the LightCycler RT-PCR. **A:** RNAs extracted from the various organs of Tg-029 mice, were reverse-transcribed into cDNA, then assayed by the LightCycler using the primers specific for β -gal and for HPRT. Copy numbers were calculated with reference to standard sample. The copy numbers of β -gal cDNA from organs were expressed as divided by those of mouse HPRT cDNA for normalization. Data were averages of three experiments. **B:** The same cDNAs were subjected to ordinary PCR using same primers. The PCR products were electrophoresed on 2% agarose gels.

RT-PCR. Almost no expression was found in organs other than the CNS in any of the transgenic lines (Figure 3A). Although expression was weakly observed using LightCycler RT-PCR in the salivary glands, kidney, and muscles in Tg-029 lines, no positive band was observed in ordinary PCR (Figure 3B). Furthermore, no positively stained cells were found in these organs by immunohistochemical staining using anti- β -gal Ab.

Expression of MCMV-e1-pro-lacZ in the CNS

Immunohistochemical analysis was performed in the CNS of the 4-week-old transgenic mice using the anti- β -gal Ab. Expression of the transgene was confined to subpopulation of neurons, as indicated by the morphology of cells and distribution of the expressed cells. Although the distribution was different in some degree among the transgenic lines, positively stained neurons were observed in the cerebral cortex, hippocampus, diencephalon, Purkinje cells in the cerebellar cortex, deep cerebellar nuclei, brainstem, and the spinal cord (Figure 4, Table 1).

In the cerebral cortex of the Tg-029 transgenic line, β -gal-positive neurons were evenly distributed throughout the cerebral layer (Figure 4, A and B). In the limbic system, β -gal-positive neurons were observed in the hippocampus and amygdala. In the hippocampus, the positive neurons were observed in the CA1 region in all of the transgenic lines (Figure 4, C and D). In the amygdala, positive neurons were observed only in the Tg-029 line (not shown). No expression was observed in the dentate gyrus in any of the transgenic lines (Table 1). In diencephalon, β -gal-positive cells were observed in the thalamus (Figure 3, E and F) and hypothalamus (not shown). In the cerebellum, expression of the transgene was observed in some Purkinje cells but not in granular cells (Figure 4, G

Figure 4. Immunohistochemical staining of expression of MCMV-e1-pro-lacZ in transgenic mice. The CNS tissues from a 4-week-old transgenic Tg-029 line were fixed in 4% PFA and embedded in paraffin. Deparaffinized sections were incubated with the anti- β -galactosidase antibody (anti- β -gal), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and then colored with 3-amino-9-ethyl carbazole (AEC). **A** and **B**: Cerebral cortex. **C** and **D**: Hippocampus dentate gyrus (DG). **E** and **F**: Diencephalon. **G** and **H**: Cerebellar cortex. Purkinje cells (arrows). **I**: Brainstem. **J**: Spinal cord. Bars, 100 μ m (**A**, **C**, **E**, **G**, **I**, and **J**); 30 μ m (**B**, **D**, **F**, and **H**).

and H). In the brainstem, positive cells were scattered in midbrain, pons, and medulla oblongata (Figure 4I). In the spinal cord, positive cells were observed in the dorsal and ventral horns (Figure 4J). A summary of the distribution of the transgene-expressing neurons in the three transgenic lines (Tg-029, Tg-041, and Tg-046) in the CNS is shown in Table 1.

Combined Staining for β -Galactosidase Activity and Immunohistochemical Staining of Neural Cell Markers

To further confirm that the MCMV-e1-pro-lacZ-expressing cells are neuronal cells, the transgene-expressing cells were double-stained immunohistochemically for neuronal and glial markers. Although neurons were not always stained with any antibody to a single neuronal marker, most of β -gal-expressing cells were double-stained with anti-NSE mAb (Figure 5A), anti- β -tubulin mAb (Figure

5B), or anti-MAP2 mAb (Figure 5C), but not double-stained with anti-GFAP Ab (Figure 5D). In addition to the evidence based on the morphology and distribution of β -gal-positive cells, these results further confirmed that the activation of the MCMV-e1-promoter in our transgenic lines was neuron-specific in the CNS.

Developmental Alterations of the Expression of MCMV-e1-pro-lacZ

To examine the expression of the transgene during development, brains of transgenic lines from E15.5 to P28 were examined immunohistochemically using anti- β -gal Ab. Positive cells were identified starting at postnatal stages except in one line (Tg-029) in which positive cells were already detected on E18.5 in the mesencephalon (not shown). The expression pattern of the β -gal-positive cells was basically the same in all of the transgenic lines: the number of β -gal-positive cells gradually increased

Table 1. Comparison of Expression of the MCMV e1-promoter-lacZ in the CNS in Transgenic Mice

Regions of the CNS	β -gal expression in transgenic mouse lines (4-week-old)		
	Tg-029	Tg-041	Tg-046
Neocortex			
Upper layers	++	+	—
Lower layers	++	+	+
Hippocampus			
CA1	++	++	++
CA3	—	+	—
Dentate gyrus	—	—	—
Amygdala	+	—	—
Thalamus	++	+	+
Hypothalamus	+	+	—
Choroid plexus	—	—	—
Cerebellum			
Purkinje's cell layer	+	+	—
Granular layer	—	—	—
Deep cerebellar nuclei	+	—	+
Brain stem	++	++	—
Spinal cord	++	—	+

Expression activity of the MCMV e1-promoter was detected by immunostaining with anti- β -gal mAb. The relative numbers of β -gal-positive cells were assessed as follows: —, negative; +, small numbers of cells were positive; ++, large numbers of cells were positive.

and the distribution spread as development proceeded. In the Tg-029 line, a small number of β -gal-expressing cells were first detected on E18.5 in the cortex (Figure 6A). In the postnatal phase, remarkable staining of positive cells was observed around the upper cerebral layer (Figure 6, B and C). The number of the β -gal-expressing cells increased and the distribution spread to all of the cortical layers (Figure 6D).

In the hippocampus, β -gal-positive cells were not detected on E18.5 (Figure 6E), and positive cells were first observed mainly in the median CA1 region of the P3 brains (Figure 6F). As the brain development proceeded, the number of the positive cells increased and the positive cells spread toward the CA3 region (Figure 6, G and H).

In the cerebellum, positive cells were first observed on E18.5 in the Tg-029 line, the expression was clearly detected in Purkinje cells and in the deep cerebellar nuclei from the postnatal stage in the Tg-029 and Tg-041 lines (not shown). In the cerebellum, the number of transgene-expressing cells did not increase markedly as development proceeded.

Enhancement of MCMV-e1-pro-lacZ Expression by Intracerebral Infection with MCMV

As the MCMV early gene e1 (M112–113) has been reported to be transactivated by IE gene expression,²⁴ as is the HCMV UL112–113 gene,³⁰ we investigated the effect of MCMV infection on the activation of the e1 promoter in the transgenic lines.

Expression of the transgene in the Tg-033 line was not detected in uninfected mice by immunohistochemical staining (Figure 7, A and E). However, when the brains were infected with MCMV within 24 hours after birth, β -gal-positive cells appeared in the cerebral cortex (Figure 7B, arrows, Figure 8 Tg-033, B) and in the hippocampus (Figure 7F, Figure 8) 6 days after infection. These β -gal-positive cells were neurons as in the other transgenic lines described above. Many virus-infected cells were observed in the same regions in the adjacent sections stained with mAb D5, which is specific for the MCMV early nuclear antigen, which is an e1 gene product (Figure 7, C and G, Figure 8, Tg-033, D). Another adjacent section stained with anti- β -gal Ab was double-stained with mAb D5 (Figure 7, D, H, arrows, Figure 8, Tg-033, C). The β -gal-expression was not always induced in virus-infected cells (Figure 7, D, H, arrowheads). However, most of β -gal-positive cells were virus-infected cells but not uninfected cells (Figure 7, D and H, Figure 8, compare Tg-033 B and C). The virus antigen was sometimes seen in the part of β -gal-positive nuclei as a dotted or punctate pattern as described previously,²¹ or seen in HCMV-infected cells stained with the antibody specific for the UL112–113 gene product.²³

Figure 5. Immunohistochemical double staining of β -galactosidase and neural markers. To confirm that β -gal-positive cells are neuronal cells, the cerebral cortex of a 4-week-old Tg-029 mouse was double-stained using anti- β -gal Ab and anti-neuron-specific enolase (NSE) Ab (**A**), anti- β -tubulin (**B**), anti-MAP2 (**C**), or anti-GFAP Ab (**D**). The brain that had been incubated with anti- β -gal Ab was colored with fast blue BB (**blue**), then incubated with antibodies specific to neural markers and colored with AEC (**red**). Bars, 30 μ m.

Figure 6. Alternation of activation of the MCMV-e1-promoter during the brain development. Expression of the transgene was examined immunohistochemically using anti- β -gal Ab in Tg-029 line at E18.5 (**A** and **E**), P3 (**B** and **F**), P7 (**C** and **G**), and P28 (**D** and **H**). In the cerebral cortex (**A–D**) and hippocampus (**E–H**), positive cells gradually increase and the distribution expanded as the growth proceeded. Bars, 100 μ m (**A–D**), 200 μ m (**E–H**).

Although a neuron singly positive for β -gal was rarely seen in nucleus (Figure 7, D, H, *), it is possible that the viral antigen may not be seen in a section of the nucleus. In the Tg-046 line, β -gal-staining neurons were observed

in the deep layer of the cortex in uninfected mice (Table 1). The number of the β -gal-positive neurons was significantly increased by MCMV infection in the cortex and hippocampus (Figure 8 Tg-046, A and B). In other trans-

Figure 7. Induction of activation of the MCMV-e1-promoter in the Tg-033 brain by MCMV infection. Intracerebral injection of the transgenic mice with MCMV (10^5 PFU) was performed within 24 hours after their birth, and the brains were examined at P7. Intracerebral injection of the same litter of the transgenic mice with MEM was also performed in the same manner for uninfected control. **A–D**: Cerebral cortex. **E–H**: Hippocampus. The brain sections were immunohistochemically stained using anti- β -gal Ab, colored with AEC (red) (**A** and **E**, uninfected; **B** and **F**, infected; arrows, β -gal-positive cells). **C** and **G**: Adjacent section of **B** and **F** were stained with mAb D5, specific to the MCMV early antigen, colored with fast blue BB (blue). **D** and **H**: The β -gal-positive cells induced by MCMV infection were (red) were double-stained with the rat mAb D5 (blue). Arrows, double-positive cells. Arrowheads, β -gal-negative-infected cells. *, cells singly positive for β -gal. Bars, 100 μ m (**A–C** and **E–G**); **D** and **H**, 30 μ m.

Figure 8. Quantitative comparison of β -gal-expressed neurons between uninfected and MCMV-infected cerebral cortex and hippocampus in the transgenic lines (Tg-029, Tg-046, and Tg-033). Transgenic mouse brains from same littermate were injected with MEM (A) and with MCMV (10^5 PFU) (B) in the same manner as Figure 7. C: β -gal-expressed neurons double-stained with mAb D5, specific for MCMV early nuclear antigen. D: Virus-infected cells stained with mAb D5. Positive cell numbers were expressed per cortex area or hippocampus of each cerebral hemisphere. The data of three animals in each transgenic line were averaged. *, $P < 0.05$ (Student's *t*-test).

genic lines (Tg-029 and Tg-041), no distinct induction of the β -gal-positive cells was detected in the cortex but induction was observed in the hippocampus after MCMV infection (Figure 8, Tg-029, A and B). The induction was somewhat different between cortex and hippocampus and was difficult to estimate in the brains of the Tg lines where the diffuse expression of the transgene had already occurred in uninfected state.

Discussion

The brain is the main target in congenital cytomegalovirus (CMV) infection²⁻⁴ and immunocompromised patients, especially AIDS patients.⁷ However, no definite evidence that CMV has a special affinity for the CNS has been published. We previously reported that the early gene e1 (M112-113) product has a tendency to be retained in neurons of MCMV-infected mouse brains,¹⁶ especially in prolonged infection.²⁰

Interestingly, the present study showed that activity of the MCMV early gene e1 promoter was restricted to the CNS in transgenic mice. Furthermore, the activation was observed only in neurons. This fact suggests that neurons of the CNS *in vivo* contain factors that drive the promoter for expression of the MCMV early gene in the absence of the viral immediate-early (IE) gene products. The MCMV early gene e1 (M112-113)²² was reported to be the counterpart of UL112-113 of HCMV^{30,31} which produces a family of four differentially spliced transcripts.³² The early gene is known to be stimulated by the HCMV IE gene products, which act as transactivators.^{30,32,33} Bühler et al²⁴ reported that expression of the MCMV early gene was also induced by the MCMV IE proteins. It is known that the cascade of expression of the early genes of CMV is induced by the IE gene products together with host cellular factors.^{14,32} The present study suggests that the MCMV early promoter can be activated in neurons of the CNS by some factors substituting for the

IE products. Mocarski et al³⁴ reported that a deletion mutant of HCMV IE1 replicated under high infective titer conditions, and they suggested that virion transactivators compensated for the lack of IE1. Therefore, it may not be necessary for the viral IE genes to be expressed for the activation of viral early gene expression in certain situations.

Neuron-specific activation of host gene promoters in transgenic mice, including the neuron-specific enolase (NSE) gene,³⁵ dopamine β -hydroxylase (DBH) gene,^{25,36} and the β 2-subunit of the neuronal nicotinic acetylcholine receptor gene³⁷ has been reported. Neuron-specific activation in transgenic mice has also been observed for viral genes such as HSV-1 IE.^{38,39} Common features of activation of neuron-specific genes in transgenic mice were as follows: the activation was observed in subpopulation of neurons but not all neurons, and was linked to neuronal maturation, although the onset of activation was observed from the embryonic stage. The activation pattern of the MCMV-e1-promoter seen in the present study was basically similar to these features. Although there was some variability in the number and distribution of the activated cells among various transgenic lines, activation of the transgene was observed in subpopulation of neurons in ubiquitous sites of the CNS, like activation of the NSE promoter.³⁵ However, in the MCMV-e1-promoter transgenic mice, expression in the granular cells of the cerebellum was not activated, and no activation was observed in organs other than the CNS, including the testes, where the NSE promoter was activated.³⁵

The transgene-expressing neurons were distributed in some layers of the cerebral cortex, part of the hippocampus, diencephalon, brainstem, Purkinje cells of the cerebellum, and spinal cord. Although the transgene-expressing neurons were observed in some degree in the embryonic stage in one of transgenic lines, activation of the promoter became more extensive postnatally in neurons of the cerebral cortex and hippocampus as development and maturation of the neurons proceeded. The number of activated cells was not correlated with the transgene copy number, in agreement with findings reported previously.^{35,38} In contrast to the activation to the MCMV IE promoter in transgenic mice,¹⁹ activation of the e1-promoter was not observed in neural progenitor cells during brain development.

In the present study, we showed that the number and extent of the activated neurons were increased in the less expressed transgenic lines by intracerebral infection with MCMV. It is possible that *in vivo* the IE gene products expressed by MCMV infection activate the e1-promoter, as observed *in vitro*.²⁴ Expression of HCMV early UL112-113 was also reported to be stimulated at least 10- to 20-fold by IE 86 protein.^{32,40} However, the enhancement of the activation of the early promoter induced by infection was not as marked in the transgenic mice as seen *in vitro* experiments. Furthermore, induction of the activation did not always occur in all of the infected cells, although the activation seemed to restrict to the infected neurons, suggesting that induction of activation of the e1-promoter occurs in virus-infected cells but not induced by extracellular factors alone in the virus infected brain. Express-

sion of the e1-promoter is different between *in vivo* and *in vitro*. This may be because the early gene is chromosomally located *in vivo*, or because the infection of neurons is less efficient than *in vitro* infection. Alternatively, some factors expressed *in vivo* may suppress the activation of the MCMV-e1-promoter.

The present evidence that activation of the MCMV-e1-promoter is restricted to neurons in transgenic mice may account, at least in part, for the special affinity of CMV for the CNS. Furthermore, neuron-specific expression of the e1 gene may cause neuronal disorders in congenital infection, and in immunocompromised patients, especially those with AIDS.⁷ In the acute phase of infection of the CNS, lytic infection of glial cells may be predominant.^{16,17} In human autopsy cases, glial cells in the periventricular zone show special susceptibility to CMV infection, sometimes with calcification.^{2,3,15} These infected cells show strong expression of the IE antigen and late antigen, and an increased level of viral DNA.¹⁶ In accordance with those findings, we reported that the MCMV IE promoter directs glial-specific expression in transgenic mice.^{18,19} In contrast, in the chronic MCMV infection of the developing mouse brain, expression of the early nuclear antigen, corresponding to the M112–113 products, was diffusely distributed in neurons of the cerebral cortex and the hippocampus.^{16,20} Since the viral DNA was not increased in these early antigen-expressing neurons, CMV may have persistently infected these neurons. It is suggested that evasion of the innate immune responses by MCMV-infected neurons may be an important factor in supporting the viral persistence in the developing brain.²⁹

It has been reported that neurotropic virus infection in the CNS tends to become persistent infection in neurons.⁴¹ CMV IE promoters of both HCMV⁴² and MCMV¹³ appear to be not well-activated in neurons when compared to glial cells. Therefore, acute phase of CMV infection of the brain, in which lytic infection of glial cells is predominant, may convert to the chronic phase of infection in which persistent infection of neurons is predominant.²⁹ Activation of the early gene e1 may be important for the transition of neurons to persistent infection. Although HCMV UL112–113 was reported to play a role in viral DNA replication in permissive cells,⁴³ the early gene E1 product in neurons may have other functions, and neuronal dysfunction or neuronal cell death may be induced by accumulation of the product in neurons. The role of the early gene E1 product in neurons in the pathogenesis of neuronal disorders in congenital CMV infection and in immunocompromised patients with AIDS remains to be elucidated.

In conclusion, using transgenic mice, we obtained here definite evidence that activation of the MCMV early e1 gene promoter was tightly restricted to subpopulation of neurons in the CNS. This fact suggests that neurons contain specific factors that drive the viral early gene and that this neuron-specific activation may be associated with persistent infection in neurons, and may cause the neuronal disorders in congenital CMV infection.

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