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The herpes simplex virus type 1 ICP0 promoter is activated by viral reactivation stimuli in trigeminal ganglia neurons of transgenic mice

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Herpes simplex virus type 1 (HSV-1) causes a latent infection in sensory ganglia neurons in humans and in the mouse model. The ability of the virus to latently infect neurons and reactivate is central to the ability of HSV-1 to remain in the human population and spread to new hosts. It is possible that neuronal transcriptional proteins control latency and reactivation by modulating activation of the HSV-1 immediate-early (IE) gene ICP0. We have previously shown that factors in trigeminal ganglia neurons can differentially activate the IE ICP0 promoter and the IE ICP4 promoter in developing trigeminal ganglia neurons of transgenic mice. Ultraviolet (UV) irradiation and hyperthermic stress have been shown to result in HSV-1 reactivation from sensory neurons in the mouse model. Reporter transgenic mice were exposed to UV irradiation or hyperthermia to test whether stimuli that are known to reactivate HSV-1 could activate viral IE promoters in the absence of viral proteins. Measurement of β-galactosidase activity in trigeminal ganglia from these transgenic mice indicated that the ICP0 promoter activity was significantly increased by both UV irradiation and hyperthermia. The IE genes ICP4 and ICP27 and the late gene gC reporter transgenes failed to be activated in parallel experiments. These results suggest that the ICP0 promoter is a target for activation by host transcription factors in sensory neurons that have undergone damage. It further suggests the possibility that activation of ICP0 gene expression by neuronal transcription factors may be important in reactivation of HSV-1 in neurons. *Journal of NeuroVirology* (2003) **9,** 336–345.

Keywords: genes, immediate-early; herpesvirus 1, human; virus latency

Introduction

Herpes simplex virus type 1 (HSV-1) initially causes a lytic infection of epithelial cells of the skin, cornea, or mucus membrane (Whitley, 2001). The virus is then transported along axons to sensory ganglia neurons where it can cause either an acute or latent infection of neurons (Stevens, 1975, 1989). The latent infection of sensory ganglia neurons allows the virus to remain in the host long term. Reactivation of latent HSV-1 with virus replication may occur periodically in humans (Whitley, 2001). The mechanism by which the latent HSV-1 genome is regulated has been the subject of much investigation (for reviews, see Fraser and Valyi-Nagy, 1993; Garcia-Blanco and Cullen, 1991; Preston, 2000; Roizman and Knipe, 2001; Wagner and Bloom, 1997). *In situ* hybridization studies have reported that gene expression from the HSV-1 genome in latently infected neurons is limited to the latency-associated transcript (LAT) gene (Croen *et al*, 1987; Deatly *et al*, 1987; Mitchell *et al*, 1990; Rock *et al*, 1987; Stevens *et al*, 1987). Subsequent studies in mice have shown that HSV-1 deletion

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mutants that do not express LATs still produce latent infections of neurons and reactivate from latency (Ho and Mocarski, 1989; Javier *et al*, 1988; Leib *et al*, 1989; Sedarati *et al*, 1989; Steiner *et al*, 1989). Studies in rabbits have shown a relationship between LATs and *in vivo* reactivation (Hill *et al*, 1990; Perng *et al*, 1996) and neuronal apoptosis (Perng *et al*, 2000).

Most theories regarding the regulation of latent and reactivated HSV-1 in sensory ganglia neurons have generally assumed that regulation by neuronal transcription factors is important (Fraser and Valyi-Nagy, 1993; Garcia-Blanco and Cullen, 1991; Tensor *et al*, 1993). Specifically, neuronal transcription factors may activate or repress HSV-1 immediate-early (IE) genes. Viral IE genes regulate their own expression and that of early and late viral genes (Cai and Schaffer, 1992; Everett, 1986, 1987; Gelman and Silverman, 1985, 1986; Mavromara-Nazos *et al*, 1986; O'Hare and Hayward, 1985; Roizman and Knipe, 2001; Zhang *et al*, 1987) and expression of all three classes of viral genes leads to HSV-1 replication. The HSV-1 ICP0 gene is thought to be the most important of the viral IE genes in the regulation of latent and reactivated infections of neurons. A number of experiments in cultured cells support this idea (Everett, 2000; Halford and Schaffer, 2001; Harris *et al*, 1989; Hobbs *et al*, 2001; Wilcox *et al*, 1997; Zhu *et al*, 1990). *In vivo* experiments utilizing HSV-1 mutants that cannot produce ICP0 also support a role for ICP0 in regulation of latent and reactivated infections in sensory neurons (Cai *et al*, 1993; Clements and Stow, 1989; Leib *et al*, 1989). However, it remains to be shown whether factors in sensory ganglia neurons *in vivo* can modulate expression from the viral ICP0 promoter following neuronal damage of the type that results in reactivation of HSV-1 from latency.

A number of stimuli have been reported to result in reactivation of latent HSV-1 from sensory ganglia neurons in the mouse model. The more common models have employed: (1) transection of the axons of latently infected sensory neurons (Walz *et al*, 1974); (2) ultraviolet (UV) light irradiation of nerve endings of latently infected sensory ganglia neurons, which innervate the cornea or skin (Blyth *et al*, 1976; Laycock *et al*, 1991; Shimeld *et al*, 1989); and (3) elevation in the core body temperature resulting in heat stress of latently infected sensory ganglia neurons (Sawtell and Thompson, 1992). Each of these methods is assumed to produce reactivation of latent HSV-1 in neurons secondary to damage to the latently infected sensory ganglia neuron.

We have previously shown that the ICP0 promoter is differentially activated in response to changes in the age of sensory neurons (Loiacono *et al*, 2002). Changes in neuronal transcription factors are presumed to be the basis for the changes in activation of the ICP0 promoter in neurons of different ages of mice. Here, we examined whether the ICP0 promoter could be activated in sensory ganglia neurons in response to neuronal damage by UV irradiation or hyperthermia. It is known from other studies that neuronal damage results in changes in host transcription factor levels in neurons (Broude *et al*, 1997; Buschmann *et al*, 1998; Gold *et al*, 1994; Herdegen *et al*, 1992, 1995; Herdegen and Leah, 1998; Jenkins *et al*, 1993; Jenkins and Hunt, 1991; Kenney and Kocsis, 1998; Leah *et al*, 1991). Our findings indicate that both UV irradiation and hyperthermia result in activation of the ICP0 promoter, but not the ICP27, ICP4, or gC promoters, in reporter transgenic mice in the absence of viral proteins. This suggests that the ICP0 promoter may be an important target for transcriptional activation during reactivation of latent HSV-1 in sensory neurons.

Results

UV irradiation of corneas and sensory nerve endings resulted in activation of the HSV-1 ICP0 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, could cause activation of the viral ICP0 promoter. HSV-1 ICP0 reporter transgenic mice (Tg6825) were exposed to UV light as described in Materials and methods and the μ-galactosidase levels in trigeminal ganglia were compared with the levels of μ-galactosidase activity in trigeminal ganglia of transgenic mice of the same genotype that were not exposed to UV light. A significant increase in ICP0 promoter activity as evidenced by an increase in μ-galactosidase levels was detected in trigeminal ganglia of the UV treated mice (Figure 1**A**). This experiment included 20 Tg6825 mice exposed to UV irradiation and 20 unmanipulated Tg6825 control mice. The above described experiment was done three times with similar results. Each of the three experiments included 12 to 20 mice in the UV-irradiated group and the same number in the unmanipulated control group. The difference in μ-galactosidase activity between the unmanipulated and UV-irradiated Tg6825 mice was statistically significant in each of the three experiments: $P < .001$ (Figure 1**A**), $P = .009$ (Figure 2**B**), and $P = .006$ (results not shown). A second line of ICP0 reporter transgenic mice (Tg3180) was tested and found to show a similar activation of the ICP0 promoter in trigeminal ganglia following UV irradiation (Figure 1**B**). Twenty unmanipulated and 20 UV-treated Tg3180 mice were used in this experiment. In parallel experiments, 20 mice from each of the following transgenic lines were exposed to UV irradiation as described while 20 mice from each transgenic line remained unmanipulated as controls: ICP27 reporter transgenic mice (Tg3058; Figure 1**C**), ICP4 reporter transgenic mice (Tg6305; Figure 1**D**), and gC reporter transgenic mice (Tg3401; Figure 1**E**). UV irradiation did not

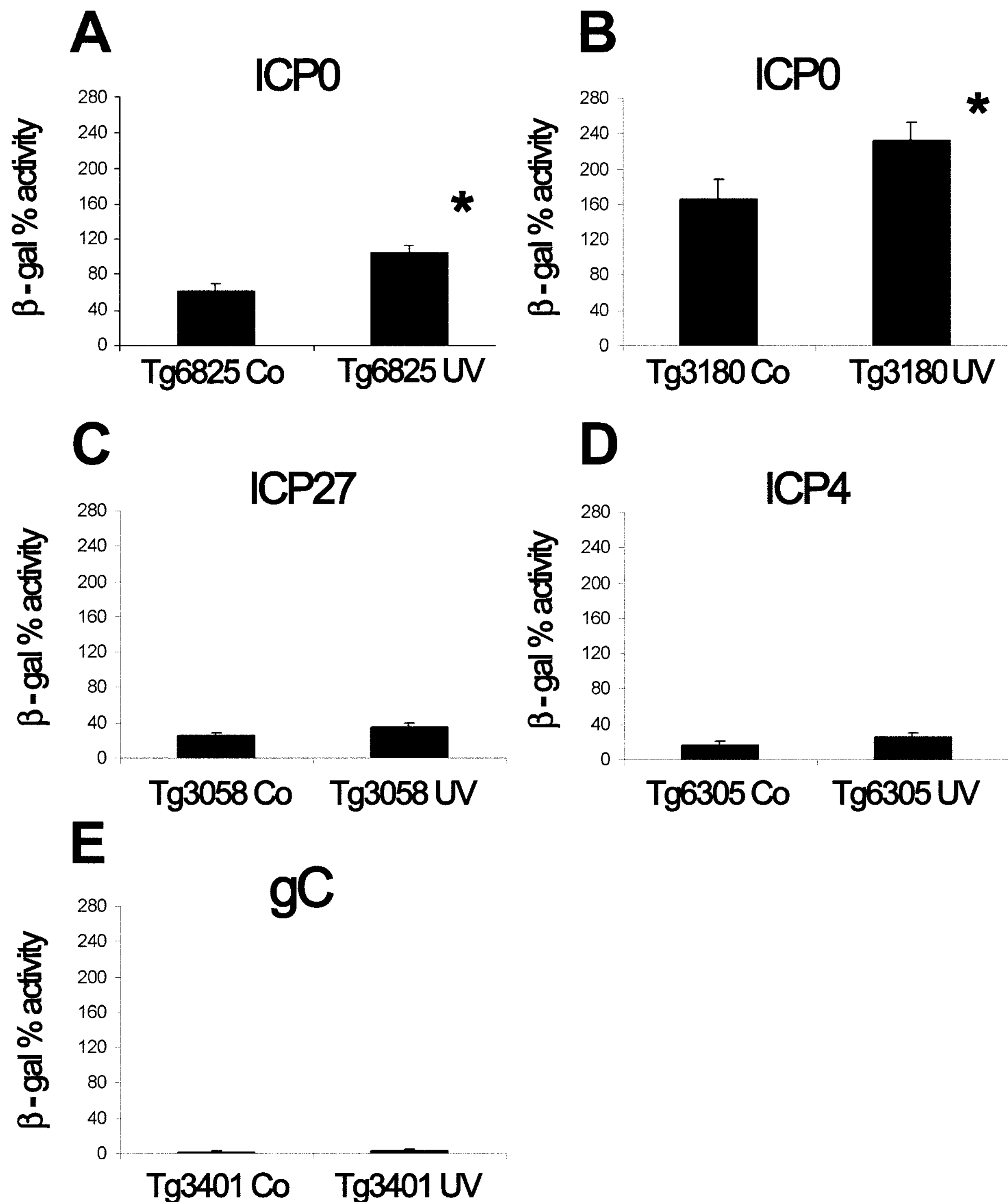

Figure 1 Ultraviolet irradiation of the cornea and sensory nerve endings induces increased expression of μ-galactosidase in neurons of the trigeminal ganglia in adult mice containing the HSV-1 ICP0 reporter transgene. The corneas and related sensory nerve endings of 20 8-week-old mice representing each transgenic line were exposed to UV light. Two days post treatment, the level of transgene expression was determined by measuring μ-galactosidase activity in the trigeminal ganglia of each mouse. These values were compared to the levels of μ-galactosidase activity in the trigeminal ganglia of each of 20 control (unmanipulated) transgenic mice from the same transgenic line. Co = groups of mice that received no treatment; UV = groups of mice that were exposed to ultraviolet light; μ-gal % activity = the percentage of μ-galactosidase activity above the level that was measured for ganglia from nontransgenic control mice. °Statistically significant difference. There was a significant increase in the activation of the ICP0 promoter in reporter transgenic mice exposed to UV irradiation but there was not a significant increase in activation of the ICP27, ICP4, or gC promoters. (**A**) Tg6825 line (ICP0-μ-galactosidase transgene), $P < .001$. (**B**) Tg3180 line (ICP0-μ-galactosidase transgene), $P = .03$. (**C**) Tg3058 line (ICP27-μ-galactosidase transgene), $P = .13$. (**D**) Tg6305 line (ICP4-μ-galactosidase transgene), $P = .30$. (**E**) Tg3401 line (gC-μ-galactosidase transgene), $P = .96$.

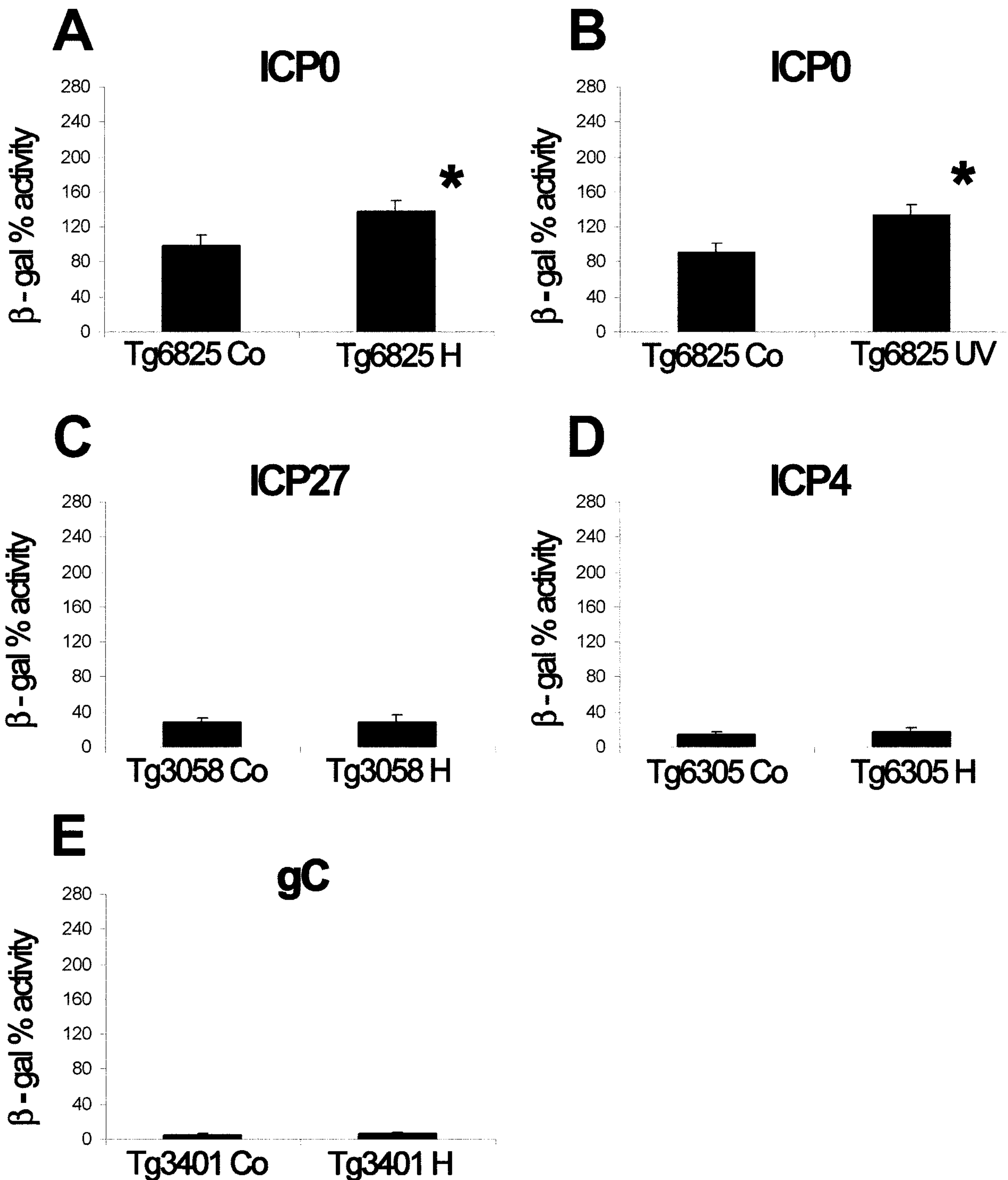

Figure 2 Hyperthermia induces increased expression of μ-galactosidase in neurons of the trigeminal ganglia in adult mice containing the HSV-1 ICP0 reporter transgene. Twenty-two 8-week-old mice representing each transgenic line were exposed to transient hyperthermia. Two days post treatment, the level of transgene expression was determined by measuring μ-galactosidase activity in the trigeminal ganglia of each mouse. These values were compared to the μ-galactosidase activity in the trigeminal ganglia of 22 control (unmanipulated) transgenic mice from the same transgenic line. As an additional control, the magnitude of activation of the ICP0 reporter transgene (Tg6825) by hyperthermia was compared with activation of the ICP0 reporter transgene by UV irradiation, shown in **B**. Co = groups of mice that received no treatment; H = groups of mice that were subjected to transient hyperthermia; UV = groups of mice that were exposed to ultraviolet light; μ-gal % activity = the percentage of μ-galactosidase activity above the level that was measured for ganglia from nontransgenic control mice. °Statistically significant difference between levels of activation of reporter transgenes. There was a significant increase in the activation of the ICP0 promoter, but there was not a significant increase in activation of the ICP27, ICP4, or gC promoters in reporter transgenic mice exposed to transient hyperthermia. (**A**) Tg6825 line (ICP0-μ-galactosidase transgene), $P = .046$. (**B**) Tg6825 line (ICP0-μ-galactosidase transgene), $P = .009$. (**C**) Tg3058 line (ICP27-μ-galactosidase transgene), $P = .47$. (**D**) Tg6305 line (ICP4-μ-galactosidase transgene), $P = .53$. (**E**) Tg3401 line (gC-μ-galactosidase transgene), $P = 0.59$.

significantly affect the activation of the HSV-1 ICP27 promoter (Figure 1**C**), ICP4 promoter (Figure 1**D**), or the gC promoter (Figure 1**E**) in trigeminal ganglia.

Hyperthermia resulted in activation of the HSV-1 ICP0 promoter in trigeminal ganglia of transgenic mice in the absence of viral proteins

To confirm that the previous results were not limited to a single type of reactivation stimulus, the effect of a second stimulus (hyperthermia) that can cause reactivation of latent HSV-1 from sensory ganglia was tested. Twenty-two ICP0 reporter transgenic mice (Tg6825) were exposed to hyperthermia and 22 unmanipulated Tg6825 mice served as controls. The ICP0 promoter was activated as measured by an increase in μ-galactosidase activity in trigeminal ganglia of Tg6825 mice that were exposed to hyperthermia (Figure 2**A**). The magnitude of the activation of the ICP0 promoter was similar whether UV irradiation or hyperthermia was used as the stimulus (Figure 2**A**, **B**). Twelve UV-treated and 12 unmanipulated Tg6825 transgenic mice were used in the experiment in Figure 2**B**. Twenty-two mice from each of the following transgenic lines were exposed to hyperthermia as previously described while 22 unmanipulated mice from each transgenic line served as controls. These lines included ICP27 reporter transgenic mice (Tg3058; Figure 2**C**), ICP4 reporter transgenic mice (Tg6305; Figure 2**D**), and gC reporter transgenic mice (Tg3401; Figure 2**E**). Hyperthermia did not significantly affect activation of the HSV-1 ICP27 promoter (Figure 2**C**), ICP4 promoter (Figure 2**D**), or gC promoter (Figure 2**E**) in trigeminal ganglia. These results demonstrate that following either UV irradiation or hyperthermia, the ICP0 promoter is activated in sensory ganglia whereas the ICP27, ICP4 and gC promoters are not activated.

The μ-galactosidase–positive cells, which were present in trigeminal ganglia of ICP0 reporter transgenic mice following UV treatment, were identified as neurons

Trigeminal ganglia of Tg6825 mice were colabeled for μ-galactosidase and a neuronal marker (neurofilament protein) as described in Materials and methods. The μ-galactosidase–positive cells in trigeminal ganglia of UV-treated and unmanipulated mice were positive for the neuronal marker (Figure 3**A**, **C**). These

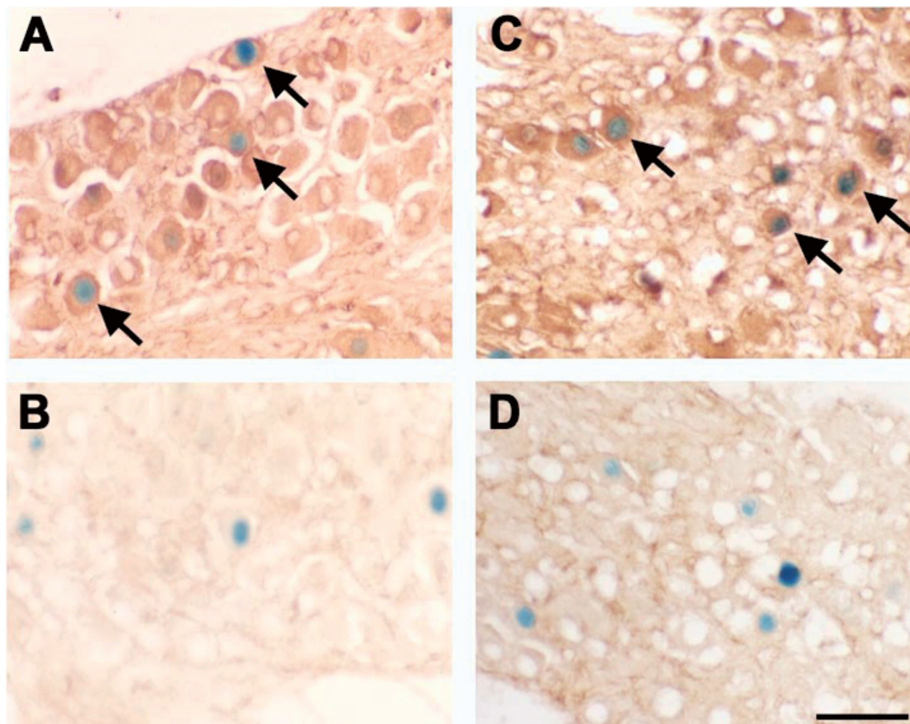

Figure 3 Colabeling of cells for μ-galactosidase and a neuronal marker (neurofilament protein) in the trigeminal ganglia of ICP0 reporter transgenic mice exposed to UV irradiation via the cornea. Sections of trigeminal ganglia from ICP0 reporter transgenic mice (Tg6825) containing μ-galactosidase–positive neurons were labeled using immuohistochemistry for the neuronal marker, neurofilament protein, as described in the text. (**A**) Section of trigeminal ganglia from an ICP0 reporter transgenic mouse that was exposed to UV irradiation. Neurons are doubly labeled for μ-galactosidase and neurofilament (*arrows*). Both neurons that express μ-galactosidase and neurons that do not express μ-galactosidase are labeled for neurofilament. (**B**) Section of trigeminal ganglia from an ICP0 reporter transgenic mouse that was exposed to UV irradiation. μ-Galactosidase–positive cells were reacted with a control monoclonal anti-bovine CD3 antibody in parallel immunohistochemical assays. (**C**) Section of trigeminal ganglia from an unmanipulated ICP0 reporter transgenic mouse. Neurons are doubly labeled for μ-galactosidase and neurofilament (*arrows*). Neurofilament-positive and μ-galactosidase–negative neurons are also present. (**D**) Section of trigeminal ganglia from an unmanipulated ICP0 reporter transgenic mouse. μ-Galactosidase–positive cells were reacted with control monoclonal anti-bovine CD3 antibody in parallel immunohistochemical assays. Bar = 40 μm.

colabeled neurons were not dispersed throughout the trigeminal ganglia but were located in clusters in a circumscribed area. Adjacent sections of trigeminal ganglia that were reacted with control antibody were negative for labeling of the neuronal marker protein (Figure 3**B**, **D**).

The increased μ-galactosidase activity in trigeminal ganglia of ICP0 reporter transgenic mice following UV irradiation was apparently not due to an increase in the number of positive neurons. The numbers of μ-galactosidase–positive cells were counted in whole trigeminal ganglia of 12 Tg6825 mice that had been UV treated (Figure 4**A**) and 12 Tg6825 mice that had not been manipulated (Figure 4**B**). There were approximately 55 positive cells per ganglia pair in the unmanipulated mice and approximately 65 positive cells per ganglia pair in UV-treated mice (Figure 4**C**). The difference was not statistically significant. These results suggest that the increased expression of μ-galactosidase in UV-treated Tg6825 trigeminal ganglia may be a result of an increase in μ-galactosidase expression in positive cells rather than an increase in numbers of positive cells. It is still possible that different subtypes of neurons are positive in UV-treated mice compared to unmanipulated mice. This question will be further investigated.

Discussion

The HSV-1 ICP0 promoter was activated in sensory ganglia neurons of reporter transgenic mice by stimuli that are known to reactivate HSV in animal models. Both UV irradiation and hyperthermia increased the level of reporter gene activity in trigeminal ganglia neurons of ICP0 reporter transgenic mice in the absence of viral proteins. No significant promoter activation was detected when transgenic mice containing the ICP27, ICP4, or gC promoter reporter genes were tested. More experiments will be required to determine whether the magnitude of ICP0 promoter activation that we observed would be sufficient to affect the regulation of HSV-1 latency and reactivation.

The most likely explanation for our findings is that neuronal damage caused by hyperthermia or UV irradiation results in modulation of transcription factors in damaged trigeminal ganglia neurons, which in turn up-regulates expression of the ICP0 reporter transgene. Changes in transcription factor levels in neurons following stimuli that cause neuronal damage are well known (Broude *et al*, 1997; Buschmann *et al*, 1998; Gold *et al*, 1994; Herdegen *et al*, 1992, 1995; Herdegen and Leah, 1998; Kenney and Kocsis, 1998; Leah *et al*, 1991). In particular, expression of genes that encode transcription factors such as c-Jun and c-Fos have been shown to be increased in response to neuronal damage. In general, it is thought that many of the changes in transcription factors are the result of initiation of the repair or regeneration response in the neuron. A well-studied example is the c-Jun gene, which is strongly up-regulated in peripheral nervous system neurons following axotomy (Broude *et al*, 1997; Kenney and Kocsis, 1998). Activation of expression of the transcription factor c-Jun has also been shown in cultured cells following UV irradiation (Devary *et al*, 1991). Corneal trauma and HSV-1 infection result in increased expression of c-Jun, c-Fos, and Oct-1 in trigeminal ganglia neurons (Valyi-Nagy *et al*, 1991). It will be important to determine which neuronal transcription factors

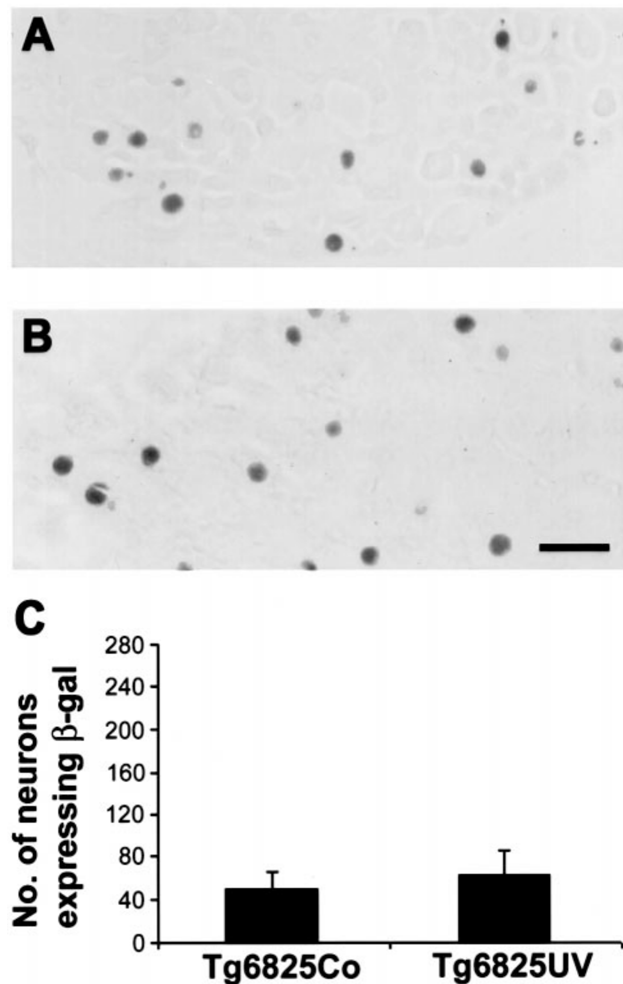

Figure 4 The numbers of μ-galactosidase–positive neurons in trigeminal ganglia from ICP0 reporter transgenic mice were not significantly different between controls and mice exposed to UV irradiation. μ-Galactosidase–positive neurons were counted in the trigeminal ganglia of ICP0 reporter transgenic mice (Tg6825) 2 days following corneal exposure to UV irradiation. These data were compared to the number of μ-galactosidase–positive neurons in unmanipulated ICP0 reporter transgenic mice. (**A**) Section of trigeminal ganglia from an ICP0 reporter transgenic mouse that was exposed to UV irradiation. Neurons are labeled for μ-galactosidase. (**B**) Section of trigeminal ganglia from an unmanipulated ICP0 reporter transgenic mouse. Neurons are labeled for μ-galactosidase. (**C**) Bar graph showing a comparison of the number of μ-galactosidase–labeled neurons in the trigeminal ganglia of ICP0 reporter transgenic mice that were either exposed to UV irradiation or unmanipulated. Results represent the means ○ standard error of the means. Bar = 25 μm.

activate the ICP0 promoter in trigeminal ganglia neurons that have undergone injury. It is not clear which sequences within the ICP0 promoter are the targets for transcriptional activators following UV irradiation or hyperthermia.

In summary, neuronal damage may activate host transcription factors, which results in expression of the HSV-1 ICP0 gene. Activation of expression of the ICP0 gene in latently infected sensory ganglia neurons could lead to reactivation of the latent HSV-1 genome. This idea does not preclude other mechanisms of regulation of the latent genome. However, this evidence suggests that a major regulatory pathway for control of reactivation of the latent HSV-1 genome targets the promoter of the ICP0 gene.

Materials and methods

Mice

Transgenic mice containing HSV-1 reporter transgenes (ICP0, ICP4, ICP27, or gC promoter fused to the *Escherichia coli* μ-galactosidase–coding sequence) used in this study were described in detail previously (Loiacono *et al*, 2002; Mitchell, 1995). The ICP0 promoter DNA fragment included nucleotides ○ 811 through +148 of the ICP0 gene. The ICP27 promoter DNA fragment included nucleotides ○ 270 through +55 of the ICP27 gene. The ICP4 promoter DNA fragment included nucleotides ○ 372 through +24 of the ICP4 gene. The gC promoter DNA fragment included nucleotides ○ 45 through +122 of the gC gene. The following transgenic lines were used: Tg6825 and Tg3180 (ICP0 reporter transgene), Tg3058 (ICP27 reporter transgene), Tg6305 (ICP4 reporter transgene), and Tg3401 (gC reporter transgene). Eight to 10-week-old transgenic and wild-type mice were used in all experiments. Heterozygous transgenic mice were generated by mating transgenic mice with wild-type (C57BL/6 ○ C3H) mice. Mice were identified as transgenic or nontransgenic for each of the reporter transgenes by polymerase chain reaction (PCR) of tail DNA (Mitchell *et al*, 1993) for the μ-galactosidase sequence (Mitchell, 1995). The primers for the μ-galactosidase coding sequence were 5′GCATCGAGCTGGGTAATAAGCGTTGGCAAT3′ and 5′GACACCAGACCAACTGGTAATGGTAGCGAC3′. All animals used in this study were maintained and handled in accordance with the *Guiding Principles in the Care and Use of Animals* (DEHEW Publication, NIH 80-23) and all experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

UV irradiation

Mice were anesthetized with isoflurane. The cornea of each eye was irradiated for 4 min using a Fotodyne UV transilluminator (302 nm wavelength). The UV transilluminator was covered except for an opening approximately the size of the mouse eye. Mice were euthanized 2 days following UV exposure and the trigeminal ganglia were dissected and removed. The ganglia were either immediately homogenized and assayed for μ-galactosidase or stored at ○ 70°C until they were used for μ-galactosidase labeling of morphologically intact cells.

Hyperthermia

Mice were placed in a water bath at 43.5°C for approximately 10 min as previously described (Sawtell and Thompson, 1992). The core body temperature of each mouse was continuously monitored throughout the procedure. The temperature of each mouse reached but did not exceed 43°C for 3 min. Animals were dried and placed in a dry incubator at 34°C for approximately 20 min following removal from the water bath. Mice were euthanized 2 days following removal from the water bath. The trigeminal ganglia were dissected from each mouse, homogenized, and assayed for μ-galactosidase.

μ-galactosidase labeling of neurons in trigeminal ganglia

For assay of μ-galactosidase labeling in intact cells, whole trigeminal ganglia were fixed in 4% paraformaldehyde for 30 min immediately following removal from ○ 70°C. 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-μ-D-galactosidase (X-gal) per ml, 120 μl of 10% Nonidet P-40, and 100 μl of 1% sodium deoxycholate per 20 ml. The X-gal was solublized in dimethylformamide and stored at ○ 20°C prior to use in the substrate solution. Ganglia were washed for 5 min in PBS. Sections of trigeminal ganglia thinly sliced with a razor blade were mounted on glass slides and coverslips were sealed with permount. The total number of μ-galactosidase–labeled neurons were counted for each pair of ganglia from each mouse in the experiment.

Assay of μ-galatosidase activity in homogenates of trigeminal ganglia

Both trigeminal ganglia from each mouse were dissected, collected in lysis buffer, and immediately homogenized with a pestle in a 1.5-ml microfuge tube. Lysis buffer contained 100 mM sodium phosphate, 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM μ-mercaptoethanol, and 0.125% Nonidet P-40. Samples were stored on ice and the 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 μ-D-galactopyranoside (Eustice *et al*, 1991; Hollon and Yoshimura, 1989). Samples were incubated for 2.5 h at 37°C and

each sample was read at 595 nm on a Beckman DU 7400 spectrophotometer. μ-Galactosidase activity was determined for each sample as the percentage of the spectrophotometer reading above nontransgenic control.

Colabeling of μ-galactosidase–positive cells for a neuron-specific marker in trigeminal ganglia of ICP0 reporter transgenic mice
Whole trigeminal ganglia from UV irradiated or unmanipulated ICP0 reporter transgenic mice 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) at 37°C for 14 to 18 h. Samples containing μ-galactosidase–positive cells were embedded in paraffin. Xylene was replaced by Clear-Rite 3 in the embedding procedure to reduce the loss of μ-galactosidase staining from cells. Six-micrometer sections of trigeminal ganglia 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 *et al*, 1993; Taus and Mitchell, 2001). 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 used at a dilution of 1:40. 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 then dehydrated and coverslipped using permount.

Statistics
The Student's t test or the Mann-Whitney rank sum test was used for statistical analysis. The level of confidence at which differences between experimental groups were judged to be significant was $P < .05$.

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