

# Depolarization Strongly Induces Human Cytomegalovirus Major Immediate-Early Promoter/Enhancer Activity in Neurons\*

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**Activity-dependent changes in gene expression involving the transcription factor cAMP-response element-binding protein (CREB) occur in learning and memory, pain, and drug addiction. This mechanism may also be important for cytomegaloviral infections of the brain. The human cytomegalovirus major immediate-early promoter/enhancer (hCMV promoter), rate-limiting for productive cytomegalovirus infection, contains five cAMP-response elements (CREs). Indirect evidence suggests that this promoter does not function in unstimulated neurons. Here we test the hypothesis that expression from the hCMV promoter in neurons is induced by membrane depolarization. For these experiments, we infected cultured sympathetic and hippocampal neurons with hCMV-green fluorescent protein (GFP) promoter/reporter constructs using adenoviral gene transfer techniques and measured transgene expression by quantifying GFP fluorescence and GFP mRNA levels. We found that depolarization up-regulates promoter activity by >90-fold. Moreover, our results from pharmacological experiments suggest that this induction occurred through a CREB-dependent pathway. Importantly, site-directed mutagenesis of all five CREs in the promoter blocked this up-regulation almost completely, whereas mutating four of them had no effect. We conclude that the hCMV promoter acts as a molecular switch in neurons and is strongly induced by membrane depolarization, neuronal activity, or other stimuli that activate CREB. These results may provide insight into molecular mechanisms of cytomegalovirus-related diseases of the brain.**

Human cytomegalovirus (hCMV)<sup>1</sup> infections, often harmless in healthy individuals, produce numerous debilitating symptoms in those with immature or impaired immune systems. In persons with AIDS, for example, cytomegalovirus infections cause severe neurological disorders (1); in infants, congenital infections cause impaired brain growth, mental retardation, and progressive motor deficits (2). Therefore, it is important to

understand the mechanisms for cytomegalovirus production in the nervous system. Productive cytomegaloviral infections require transcription of immediate-early genes in the viral genome (3, 4). Expression of these genes is controlled by the hCMV major immediate-early promoter/enhancer (hCMV promoter) (5). As activity of this promoter is rate-limiting for productive viral infection, many groups have studied how this promoter functions to understand the molecular mechanisms of cytomegalovirus infections. The hCMV promoter is highly enriched with a densely packed array of *cis*-elements (3) and is among the strongest known promoters in mammalian cells. As such, it is commonly used to drive the expression of foreign genes in a wide variety of cell types.

Surprisingly, the hCMV promoter appears not to function well in neurons. For example, studies using primary cortical cultures indicate that the hCMV promoter expresses transgenes well in glia but not in neurons (6). Furthermore, in mice transgenic for hCMV promoter-reporter constructs, only a few neurons express the transgene (7, 8), indicating that hCMV promoter activity is low in most neurons. That a few neurons express the transgene at high levels suggests that neurons regulate promoter activity.

In preliminary experiments on neurons expressing foreign genes under the control of the hCMV promoter, we observed that depolarization induced a dramatic up-regulation of transgene expression suggesting that activity-dependent mechanisms modulate hCMV promoter function. Consistent with this idea, the hCMV promoter contains a number of *cis*-elements that bind transcription factors thought to be regulated by neuronal activity; these include, five cAMP response elements (CRE), four NF- $\kappa$ B-binding sites, an AP-1 site, and a serum-response element (SRE) (3).

Because we are interested in the molecular mechanisms underlying activity-dependent gene expression, and because activity-dependent mechanisms may lead to the progression of hCMV infections in the brain, we furthered our preliminary findings to determine if depolarization regulates the hCMV promoter. In addition, since elevating cAMP in non-neuronal cell lines increases hCMV promoter function (9–13), we examined the role of the five CREs in the promoter. Our results show that hCMV promoter activity in neurons is up-regulated >90-fold by depolarization. Site-directed mutagenesis of the CREs in the promoter blocked the depolarization-induced expression by over 95% indicating that the CREs mediate this induction.

## EXPERIMENTAL PROCEDURES

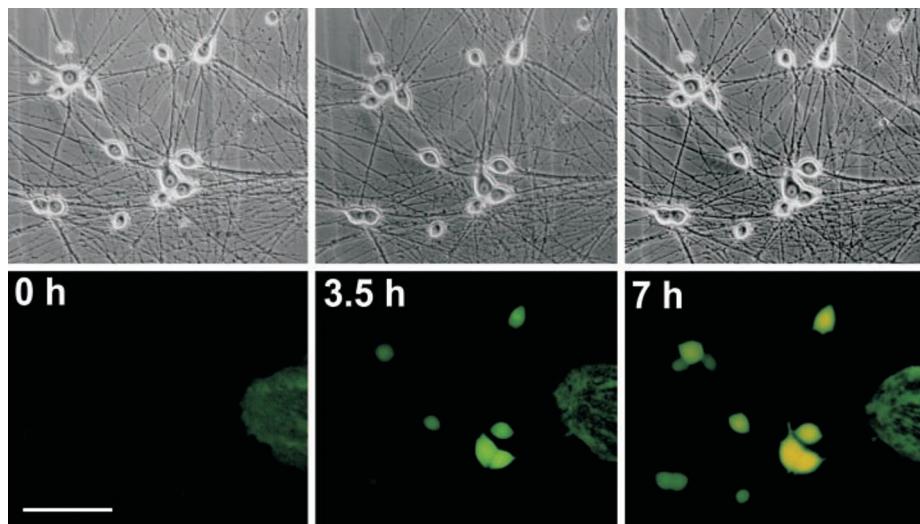
**Sympathetic Neuron Cultures**—We cultured superior cervical ganglia (SCG) neurons from 1-day-old Sprague-Dawley rats (Charles River, Canada) as described previously (14), except we used trypsin (180 units/ml, Worthington) for 30 min instead of dispase/collagenase. In some experiments, we centrifuged the cell suspension through a Percoll gradient (Amersham Pharmacia Biotech) to decrease the relative contribution of non-neuronal cells to these cultures. In experiments where

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<sup>1</sup> The abbreviations used are: hCMV, human cytomegalovirus; CaMK, calcium/calmodulin-dependent protein kinase; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; GFP, green fluorescent protein; MAP-2, microtubule-associated protein 2; NMDA, N-methyl-D-aspartate; SCG, superior cervical ganglia; bp, base pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; APV, (R)-(-)-2-amino-5-phosphono valeric acid.

**FIG. 1. Depolarization induces GFP fluorescence in sympathetic neurons infected with adenoviral vectors containing hCMV-GFP reporter constructs.** SCG neurons were infected overnight with an adenovirus containing an hCMV-GFP reporter construct at day 5 *in vitro* and stimulated with 40 mM K<sup>+</sup> 2 days later. The same field was photographed at 0, 3.5, and 7 h after stimulation. The top panels are phase-contrast micrographs, and the bottom panels are the corresponding fluorescent images. The letters cH, photo-etched on the coverslip, are visible in the background of the phase-contrast micrographs. Scale bar, 140  $\mu$ m.



we did not take this precaution, the fold increase in GFP mRNA induced by depolarization was significantly less. Cultures were fed every 3 days and treated with cytosine arabinoside (10  $\mu$ M; Sigma) from days 2 to 5. In Fig. 1, we used photo-etched coverslips (Bellco, Fayetteville, NY).

**Hippocampal Neuron Cultures**—We dissociated hippocampi from 3- to 4-day-old rats using papain (Worthington) (15), and we plated the cells on laminin/poly-D-lysine-coated aclar coverslips in Neurobasal Medium (Life Technologies, Inc.) with B-27 (Life Technologies, Inc.), penicillin, streptomycin, and L-glutamine (Life Technologies, Inc.). The cells were kept at 37 °C in 5% CO<sub>2</sub>. Half the medium was replaced every 3 days. We included L-glutamic acid (25  $\mu$ M; Sigma) for the first 4 days and cytosine arabinoside (5  $\mu$ M; Sigma) from days 2 to 5.

**Virus Production**—Mutations were made sequentially in the hCMV promoter of pEGFP-C1 (CLONTECH) with the QuickChange Kit (Stratagene). The polylinker of wild-type or mutated pEGFP-C1 was removed, and the CMV-GFP-poly(A)<sup>+</sup> cassette was ligated into pShuttle (16). Replication-deficient viral constructs were made according to He *et al.* (16).

**Viral Titration, Infection of Cultures, and Stimulation of Cells**—Viruses were titrated in duplicate with the cytopathic effect method (17). Titors of all mutated viral constructs were conducted in parallel with the same wild-type virus to ensure that titer determinations were accurate and consistent. We infected SCG cultures at day 5 and hippocampal cultures at day 8 with 50 infectious particles/ $\mu$ l for GFP fluorescence experiments and with 5–20 infectious particles/ $\mu$ l for mRNA experiments. In most experiments, we infected cultures overnight, rinsed them twice with virus-free medium the next morning, and stimulated them for 24 h. In the experiment shown in Fig. 1, after the rinsing step we incubated the cultures in virus-free medium for 48 h before stimulating them with high K<sup>+</sup>.

**Immunocytochemistry and Imaging**—Hippocampal cultures were fixed in 4% paraformaldehyde, permeabilized in 100% methanol, and sequentially incubated in a 1/10,000 dilution of an anti-MAP-2 antibody (HM-2; Sigma), biotin-conjugated goat anti-mouse IgG (3  $\mu$ g/ml; Jackson ImmunoResearch), and streptavidin-Cy3 (5  $\mu$ g/ml; Jackson ImmunoResearch). We captured images of hippocampal cultures with a Bio-Rad MicroRadiane confocal microscope. We viewed live sympathetic neurons using a Zeiss IM 35 microscope with a 40 $\times$  objective (Plan-Neofluar, N.A. 0.75) and photographed them with 35-mm film (Gold-Max 800, Eastman Kodak Co.). To quantify GFP fluorescence, we used a CCD camera (CoolSnap HQ, Roper Scientific) and ImageQuaNT software (Molecular Dynamics).

**RNase Protection Assay**—RNA was extracted with the RNeasy kit (Qiagen). Assays were done according to Krieg and Melton (18) with minor modifications (19). The GFP probe template was produced by performing polymerase chain reaction on pEGFP-C1 (CLONTECH) using the following oligonucleotides: 5'-GGAGGACGGCAACATCC-3' and 5'-CTGCTGGAGTTCTGTGACC-3'. <sup>32</sup>P-Labeled riboprobes for GFP and GAPDH were hybridized overnight with 0.2–0.5  $\mu$ g of total RNA, treated with RNase, and run on polyacrylamide gels. Results were quantified with a PhosphorImager and ImageQuaNT software (Molecular Dynamics).

## RESULTS

**hCMV Promoter Activity in Sympathetic Neurons**—To examine hCMV promoter function, we expressed an hCMV-GFP promoter/reporter construct in primary cultured neurons; this construct contained the first 589 bp of the human cytomegalovirus major immediate-early promoter/enhancer (hCMV promoter) driving the expression of green fluorescent protein (GFP). With conventional techniques, such as calcium phosphate or lipid-mediated transfection, we achieved poor transfection efficiencies (<0.3% for sympathetic neurons; data not shown). Therefore, we used adenoviral gene transfer (20); this approach gave a high infection efficiency (at least 95%) in primary neonatal sympathetic and hippocampal cultures.

We infected cultures of neonatal rat superior cervical ganglia (SCG) after 5 days *in vitro* with adenoviruses containing the hCMV-GFP reporter. These cultures are highly enriched for neurons. Within hours after infection, most of the 20–40 non-neuronal cells in each culture were GFP-positive, whereas few of the 1000–2000 neurons were GFP-positive, even at 48 h post-infection. However, we found that treating cultures with high K<sup>+</sup> (40 mM) induced GFP expression in most neurons. Fig. 1 shows an example where we followed 13 infected neurons after exposure to high K<sup>+</sup>. Two days after infection, no neurons were GFP-positive, although the single non-neuronal cell in the field fluoresced; however, after 3.5 h in high K<sup>+</sup>, 6/13 neurons clearly expressed GFP, and after 7 h, 11/13 neurons were GFP-positive. In high K<sup>+</sup>-treated cultures, we could detect GFP-positive neurons within ~2 h of stimulation, and the fluorescence intensity increased steadily with time. Following the removal of high K<sup>+</sup>, neurons remained GFP-positive for at least 3–5 days without obvious effects on neuronal growth, differentiation, or electrophysiological properties (data not shown).

To measure the kinetics of GFP induction, we determined the fluorescence intensity of living neurons over time using a CCD camera. We infected cultures overnight at day 5 *in vitro* and then stimulated with high K<sup>+</sup>. Fig. 2A shows an example where we followed 4 neurons over the first 9.5 h in high K<sup>+</sup>. After a lag of 2–3 h, GFP fluorescence intensity increased steadily over time. To quantify this GFP induction by high K<sup>+</sup>, we counted the proportion of GFP-positive neurons 24 h after stimulation (Fig. 2B). In control medium, 5% of the neurons were GFP-positive, whereas after high K<sup>+</sup> treatment, over 90% were GFP-positive ( $p < 0.0001$ , Student's *t* test;  $n = 6$ ). Digital quantification showed that cell counts underestimate the re-

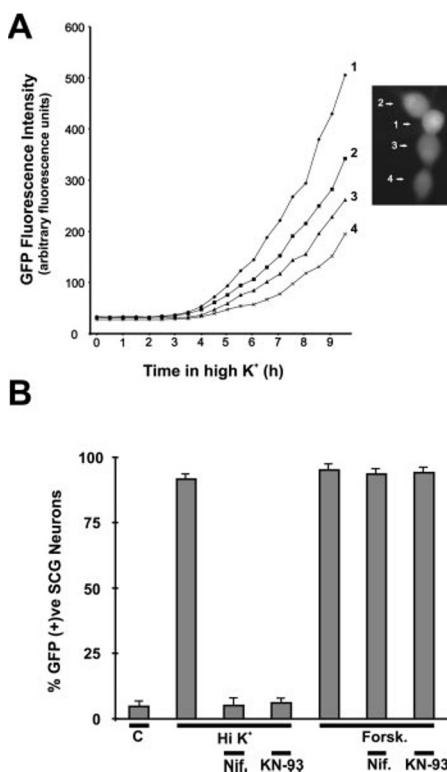
sponse to high  $K^+$ ; not only did high  $K^+$  increase the proportion of GFP-positive neurons, it increased the amount of green fluorescence per positive cell as well.

In neurons, depolarization opens L-type  $Ca^{2+}$  channels and leads to activation of calcium/calmodulin-dependent protein

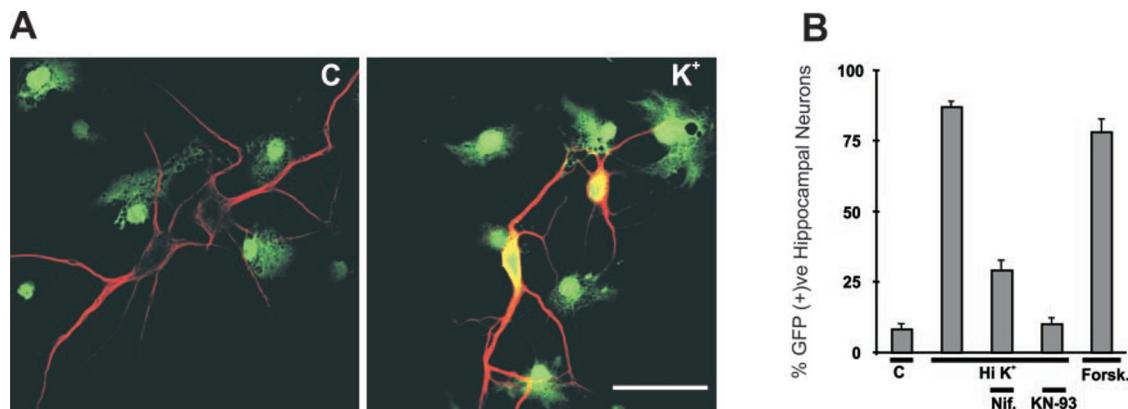
kinase (CaMK) and subsequent phosphorylation of the cAMP-response element-binding protein (CREB). Activation of CREB, or related family members, results in expression of genes containing cAMP-response elements (CREs) in their promoters (21). As the hCMV promoter contains 5 CREs (3), we tested the role of L-type  $Ca^{2+}$  channels and CaMK in the induction of GFP by depolarization. Both nifedipine ( $5 \mu M$ ), an L-type  $Ca^{2+}$  channel blocker, and KN-93 ( $10 \mu M$ ), a CaMK inhibitor, prevented high  $K^+$ -induced GFP expression (Fig. 2B). To determine whether other stimuli that activate CREB could induce expression from the hCMV promoter, we activated cAMP-dependent protein kinase with forskolin. We found that forskolin ( $10 \mu M$ ) also induced GFP expression in over 90% of the neurons; this induction was not affected by nifedipine or KN-93 (Fig. 2B).

To demonstrate that high  $K^+$  treatment activated CREB, we immunostained SCG neurons with an anti-phospho-CREB antibody. We observed strong nuclear staining of phospho-CREB within 7.5 min; nuclei remained phospho-CREB-positive for at least 24 h in high  $K^+$  (data not shown). Moreover, nifedipine ( $5 \mu M$ ) and KN-93 ( $10 \mu M$ ) blocked high  $K^+$ -induced CREB phosphorylation. Together, these results suggest that stimuli that activate CREB induce gene expression from the hCMV promoter.

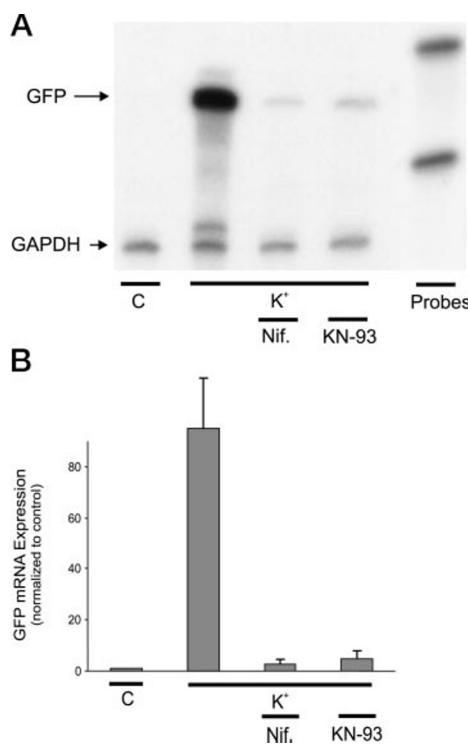
**hCMV Activity in Hippocampal Neurons**—Since activity in hippocampal neurons leads to CREB-mediated gene expression (22, 23), we reasoned that depolarization should induce GFP expression from the hCMV promoter in infected primary cultured hippocampal neurons, as we observed with sympathetic neurons. To test this, we monitored GFP expression in hippocampal neurons that were infected with the hCMV-GFP construct. As these cultures contain a large number of non-neuronal cells, we distinguished neurons from non-neuronal cells with an antibody against the neuron-specific protein, MAP-2. Adenoviruses containing the hCMV-GFP promoter/reporter construct infected both neurons and non-neuronal cells with high efficiency. Fig. 3A shows neonatal hippocampal cultures after 10 days *in vitro* which had been infected overnight, washed, and then treated for 24 h with high  $K^+$  or control medium. In both conditions, we detected GFP in non-neuronal cells. However, in control medium, few infected hippocampal neurons expressed GFP; whereas treating these cultures with high  $K^+$  ( $20 \text{ mM}$ ) induced GFP expression in the majority of neurons. Like sympathetic neurons, GFP induction was largely prevented by nifedipine ( $5 \mu M$ ) and KN-93 ( $10 \mu M$ ) and was



**FIG. 2. Depolarization-induced GFP expression is blocked by nifedipine and KN-93 and mimicked by forskolin.** SCG cultures were infected overnight at day 5 *in vitro* with an adenovirus containing an hCMV-GFP reporter construct. *A*, GFP fluorescence was measured in 4 neurons with a CCD camera every 30 min for 9.5 h after high  $K^+$  stimulation. The CCD image at 6 h is shown on the right. *B*, The percentage of GFP-positive neurons was determined after treating the cultures for 24 h with control medium (*C*), 40 mM  $K^+$  (*High K<sup>+</sup>*), 40 mM  $K^+$  +  $5 \mu M$  nifedipine (*Nif.*), 40 mM  $K^+$  +  $10 \mu M$  KN-93 (*KN-93*), 10  $\mu M$  forskolin (*Forsk.*), 10  $\mu M$  forskolin +  $5 \mu M$  nifedipine, and 10  $\mu M$  forskolin +  $10 \mu M$  KN-93. Each experiment was done in duplicate or triplicate. The data are averages of 4–6 experiments, and a total of 800–1200 neurons was counted in each condition. Error bars indicate  $\pm$  S.E.



**FIG. 3. Depolarization induces GFP expression in cultured hippocampal neurons infected with adenoviral vectors containing hCMV-GFP reporter constructs.** *A*, an example of hippocampal cultures infected overnight at day 8 *in vitro* with adenoviral vectors containing an hCMV-GFP reporter construct. The cultures were treated for 24 h with control medium (*C*) or 20 mM  $K^+$  (*K<sup>+</sup>*), fixed, and stained with an antibody against MAP-2 (red). GFP (green) is present in glia in both conditions, but neurons are GFP-positive only after high  $K^+$  stimulation. Scale bar, 50  $\mu M$ . *B*, shows the percentage of GFP-positive hippocampal neurons when treated for 24 h after infection with control medium (*C*), 20 mM  $K^+$  (*Hi K<sup>+</sup>*), 20 mM  $K^+$  +  $5 \mu M$  nifedipine (*Nif.*), 20 mM  $K^+$  +  $10 \mu M$  KN-93 (*KN-93*), or 10  $\mu M$  forskolin (*Forsk.*). Each experiment was done in duplicate or triplicate. The data are averages of 4–6 experiments, and a total of 800–1200 neuron was counted in each condition. Error bars indicate  $\pm$  S.E.

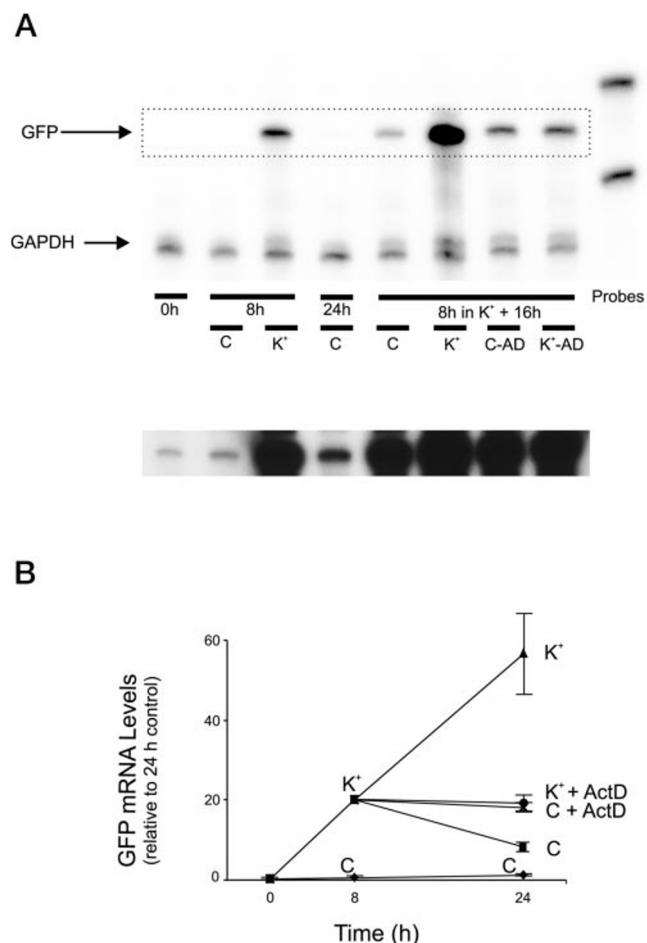


**FIG. 4. GFP mRNA is highly induced by depolarization.** *A*, RNase protection assay showing GFP and GAPDH mRNA levels from SCG cultures infected overnight at day 5 *in vitro* with an adenovirus containing an hCMV-GFP reporter construct. The cultures were treated for 24 h with control medium (*C*), 40 mM K<sup>+</sup> (*K'*), 40 mM K<sup>+</sup> + 5 μM nifedipine (*Nif.*), or 40 mM K<sup>+</sup> + 10 μM KN-93 (*KN-93*). *B*, relative GFP mRNA expression quantified from 4 to 7 separate experiments like those shown in *A*. Data are normalized to control. Error bars indicate ± S.E.

mimicked by forskolin (10 μM) (Fig. 3*B*); interestingly, unlike sympathetic neurons, the block by nifedipine was not as great as that by KN-93.

**Depolarization Induces Transcription from the hCMV Promoter**—By counting GFP-positive neurons, we obtained a quantitative measure of the proportion of neurons that expressed the transgene at levels high enough to be detected by the eye. In both sympathetic and hippocampal cultures, depolarization with high K<sup>+</sup> not only increased the proportion of GFP-positive neurons but also increased the average fluorescence in these GFP-positive neurons. To obtain a more direct and quantitative measure of GFP expression in neurons, we determined GFP mRNA levels using RNase protection assays. For these experiments we used SCG cultures; these cultures were highly enriched for neurons, and the amount of RNA from the few non-neuronal cells made only a small contribution to total RNA. These measurements of gene induction specifically in neurons are far more difficult in neonatal hippocampal cultures where the ratio of non-neuronal cells to neurons is much higher.

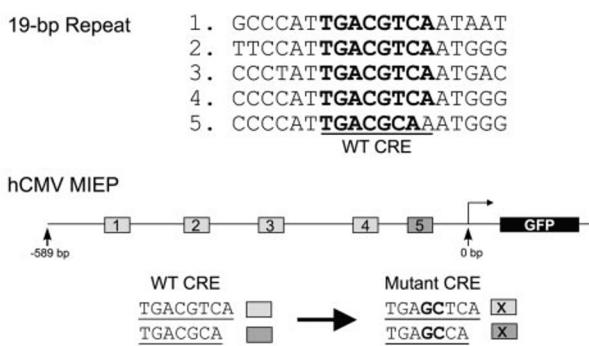
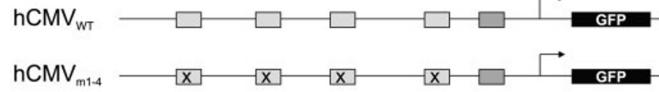
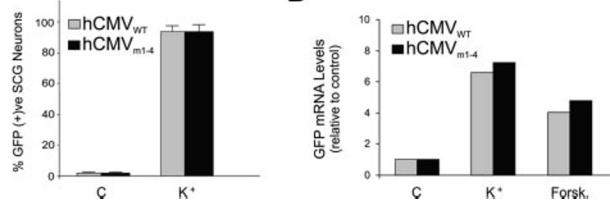
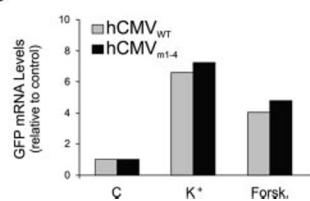
Fig. 4*A* shows an example of GFP mRNA levels from cultures infected overnight with the hCMV-GFP construct and then treated for 24 h under four different conditions as follows: control, high K<sup>+</sup> (40 mM), and high K<sup>+</sup> with nifedipine (5 μM), or high K<sup>+</sup> with KN-93 (10 μM). Compared with control, high K<sup>+</sup> induced a 93.4 ± 18.8-fold increase in GFP mRNA in 24 h; this increase was blocked by both nifedipine and KN-93 (Fig. 4*B*). The increase in GFP mRNA due to high K<sup>+</sup> stimulation continued approximately linearly for up to 55 h (data not shown). In control conditions, a portion of the total GFP mRNA came from non-neuronal cells; consequently, the fewer the non-neuronal cells, the lower the base line. As a result, we obtained



**FIG. 5. Effects of actinomycin D on depolarization-induced expression of GFP mRNA.** *A*, an example of an RNase protection assay showing GFP mRNA from SCG neurons infected overnight at day 5 *in vitro* with an adenovirus containing an hCMV-GFP reporter construct and examined at 0 (*1st lane*), 8 (*2nd and 3rd lanes*), and 24 h (*4th to 8th lanes*). Some cultures were maintained in control medium (*C*) for 24 h. Others were treated with 40 mM K<sup>+</sup> initially for 8 h; for the next 16 h, the cultures were treated with high K<sup>+</sup>, alone or with actinomycin D (*K'-AD*), or returned to control medium, alone or with actinomycin D (*C-AD*). GAPDH was used as an internal control. The inset below shows a longer exposure of the marked region. *B*, quantification of 3 experiments like that shown in *A*. Data are normalized to the values at 8 h in high K<sup>+</sup> and shown relative to GFP mRNA levels after 24 h in control medium. Error bars indicate ± S.E.

the largest fold increase (up to 150-fold in 24 h) in cultures containing the fewest non-neuronal cells; in cultures with more non-neuronal cells the fold increase was less.

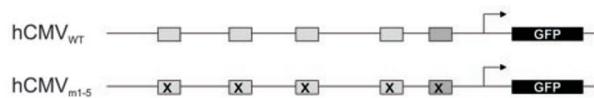
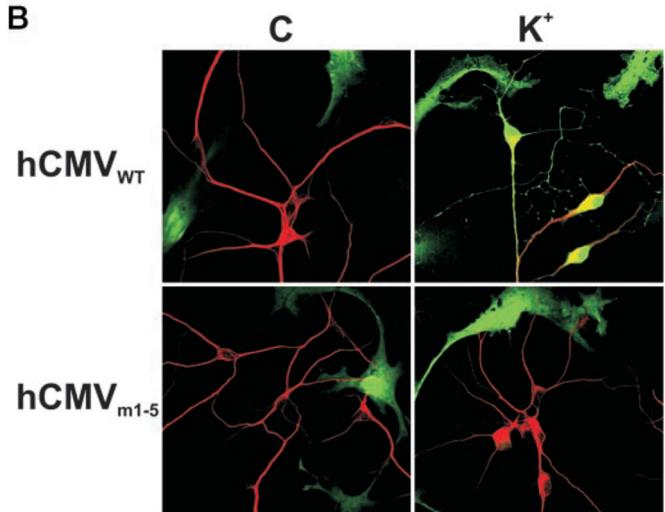
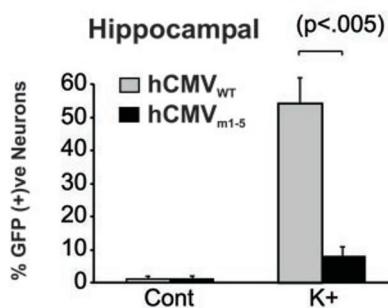
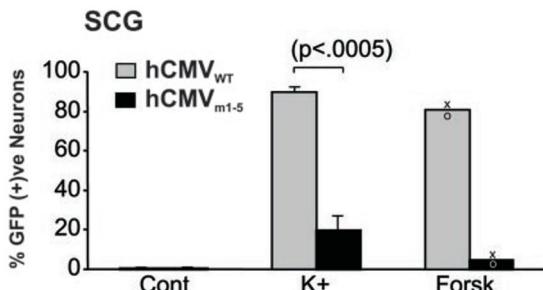
It seems likely that most of this increase in GFP mRNA induced by high K<sup>+</sup> resulted from an increase in transcription from the hCMV promoter; however, some of the increase may have resulted from increased GFP mRNA stability. For example, depolarization stabilizes mRNA for an N-type calcium channel subunit in cultured SCG neurons (24). To get an idea of the relative contribution of transcription *versus* mRNA stability, we blocked transcription pharmacologically with actinomycin D. We infected sympathetic neurons overnight with the hCMV-GFP construct, and 1 day later we treated them with high K<sup>+</sup> or left them in control medium. After 8 h, the high K<sup>+</sup>-treated dishes were divided into two groups: one continued with high K<sup>+</sup>, and the other was returned to control medium. In each group, half the dishes were treated with actinomycin D; this produced four different conditions in addition to those left in control medium throughout: 40 mM K<sup>+</sup> (high K<sup>+</sup>) or 5 mM K<sup>+</sup> with or without actinomycin D (4 μM). We measured GFP

**A****B****C****D**

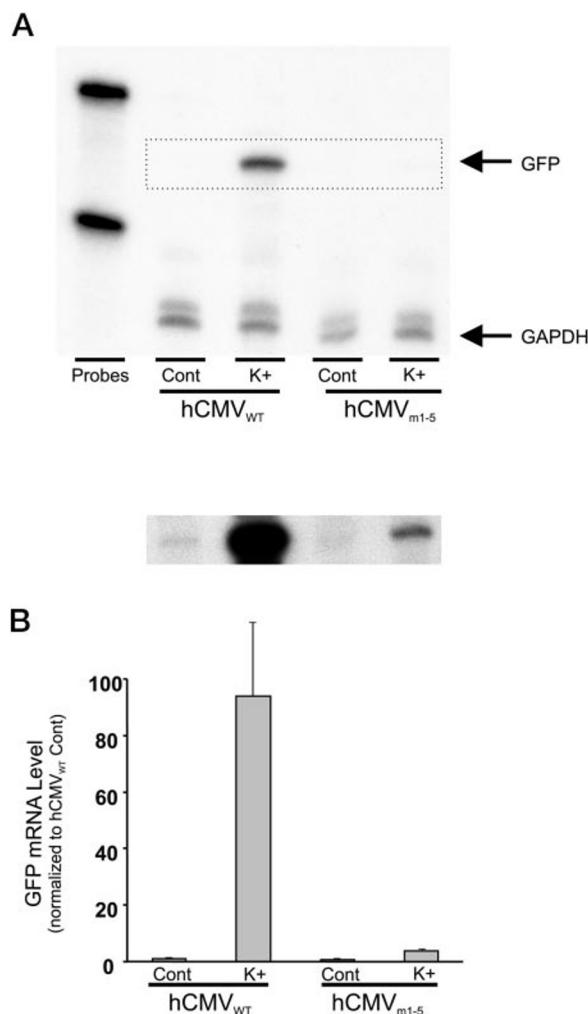
**FIG. 6. Mutation of the four consensus CREs does not affect hCMV promoter induction by depolarization.** *A* shows the sequence of the five 19-bp repeats in the hCMV promoter. 1–4 show the four consensus CREs, and 5 shows the non-consensus CRE. Below is a schematic arrangement of the five 19-bp repeats in the hCMV-GFP reporter construct and how the CREs were mutated. *B*, schematic showing the wild-type hCMV-GFP reporter (hCMV<sub>WT</sub>) and the one with the four consensus CREs mutated (hCMV<sub>m1-4</sub>). *C* shows the percentage of SCG neurons expressing GFP when infected with hCMV<sub>WT</sub> or hCMV<sub>m1-4</sub>. Neurons were treated with control medium (*C*) or 40 mM K<sup>+</sup> (*K<sup>+</sup>*) and counted as in Fig. 2. Each experiment was done in duplicate, and the data are an average of 4 experiments. More than 800 neurons were counted in each condition. Error bars indicate  $\pm$  S.E. *D*, quantification of an RNase protection assay performed on RNA from SCG neurons infected with hCMV<sub>WT</sub> or hCMV<sub>m1-4</sub> and treated with control medium, 40 mM K<sup>+</sup> (*K<sup>+</sup>*), or 10  $\mu$ M forskolin (*Forsk.*). The fold increase in this experiment was less than usual because these cultures contained a relatively high proportion of non-neuronal cells.

mRNA with RNase protection assays at 0, 8, and 24 h. An example of one of these experiments is shown in Fig. 5A. In the absence of high K<sup>+</sup> stimulation, GFP mRNA levels were low and increased a small amount over time (Fig. 5). It is likely that much of this GFP mRNA comes from non-neuronal cells. At 8 h we measured a large increase in GFP mRNA in cultures treated with high K<sup>+</sup>. Over the next 16 h, GFP mRNA levels continued to increase in high K<sup>+</sup>. Importantly, this increase in mRNA between 8 and 24 h was completely blocked by actinomycin D (Fig. 5). Moreover, in the presence of actinomycin D, GFP mRNA levels in high K<sup>+</sup>-treated cultures were not significantly different from those returned to control (5 mM K<sup>+</sup>) medium for 16 h, indicating that high K<sup>+</sup> has little effect on GFP mRNA stability. Taken together, these results suggest that the large increase in GFP mRNA induced by depolarization resulted from increased transcription from the hCMV promoter. As an aside, we noticed GFP mRNA levels in cells returned to 5 mM K<sup>+</sup> for 16 h without actinomycin D were lower than those treated similarly but with actinomycin D; this suggests that a transcription-dependent factor(s) destabilizes GFP mRNA. We did not investigate this further.

*Induction of the hCMV Promoter by Depolarization Requires*

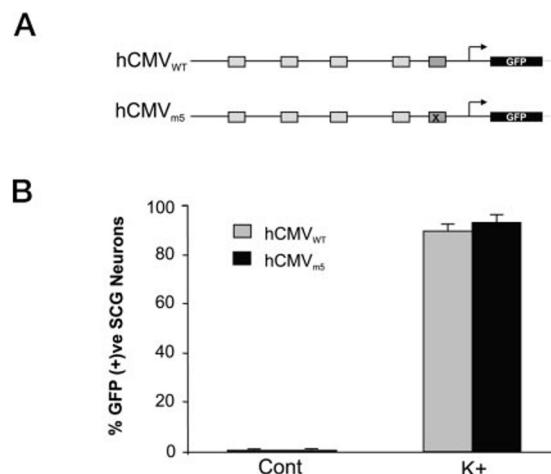
**A****B****C****D**

**FIG. 7. CREs mediate depolarization-induced GFP expression from the hCMV promoter.** *A*, schematic showing the wild-type hCMV-GFP reporter (hCMV<sub>WT</sub>) and one with all five CREs mutated (hCMV<sub>m1-5</sub>). *B*, shown are photomicrographs of hippocampal cultures infected overnight at day 8 *in vitro* with an adenovirus containing either hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub>, treated with control medium (*C*) or 20 mM K<sup>+</sup> (*K<sup>+</sup>*) for 24 h, and fixed and stained for MAP-2 (red) (see Fig. 3). *C* shows the percentage of hippocampal neurons expressing GFP when infected with hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub>. Neurons were counted as in Fig. 3. Each experiment was done in duplicate, and the data are an average of 4 experiments. Over 800 neurons were counted in each condition. Error bars indicate  $\pm$  S.E. *D* shows the percentage of SCG neurons expressing GFP when infected with hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub>. Neurons were treated with control medium (*Cont*), 40 mM K<sup>+</sup> (*K<sup>+</sup>*), or 10  $\mu$ M forskolin (*Forsk*) and counted as in Fig. 2. Each experiment was done in duplicate, and the data are an average of 4 experiments except for forskolin treatment, which was done only twice, and the data from each experiment are indicated by *X* and *O*. At least 200 neurons were counted per experiment. *C* and *D*, the error bars indicate  $\pm$  S.E., and the *p* values were calculated from Student's *t* test.



**FIG. 8. Mutation of the 5 CREs eliminates depolarization-induced transcription from the hCMV promoter.** *A* shows an RNase protection assay of GFP mRNA from SCG neurons infected overnight at day 5 *in vitro* with an adenovirus containing either hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub> and treated with control medium (*Cont*) or 40 mM K<sup>+</sup> (K<sup>+</sup>) for 24 h. GAPDH was an internal control. Below is shown a longer exposure of the marked region. *B* shows the quantification of 4 experiments identical to that shown in *A*. GFP mRNA levels were normalized to levels in control medium with hCMV<sub>WT</sub>. Error bars indicate  $\pm$  S.E.

**cAMP-response Elements**—Our results suggest that depolarization dramatically increases transcription from the hCMV promoter and that this increase involves CREB or a related family member. The hCMV promoter contains five palindromic *cis*-elements referred to as 19-bp repeats, which bind CREB or CREB family members (3, 13); four contain consensus CREs, and the fifth contains a non-consensus CRE (Fig. 6A). To investigate directly the role of these CREs in the high K<sup>+</sup>-mediated increase in hCMV promoter activity, we built a series of adenoviruses containing reporter constructs with mutated CREs and monitored GFP expression in neurons. First, we tested the role of the 4 consensus CREs. Briefly, we altered the 8-bp palindromic CRE sequence, TGACGTCA, by reversing the middle CG (Fig. 6, *A* and *B*); the resulting sequence, TGAGCTCA, is no longer capable of binding CREB (25). Surprisingly, disrupting all 4 consensus CREs (hCMV<sub>m1-4</sub>) had no effect on high K<sup>+</sup>-induced GFP expression: in SCG cultures infected overnight with adenoviruses containing either the wild-type promoter (hCMV<sub>WT</sub>) or hCMV<sub>m1-4</sub>, a 24-h high K<sup>+</sup> treatment induced GFP expression in over 90% of the neurons (Fig. 6C). To quantify this further, we measured GFP mRNA; we observed no significant difference between cultures infected



**FIG. 9. Mutation of the fifth non-consensus CRE has no effect on hCMV promoter induction by depolarization.** *A* shows a schematic of the wild-type hCMV-GFP reporter (hCMV<sub>WT</sub>) and the one with only the fifth consensus CRE mutated (hCMV<sub>m5</sub>). *B* shows the percentage of SCG neurons expressing GFP when infected with hCMV<sub>WT</sub> or hCMV<sub>m5</sub>. Neurons were treated with control medium (*Cont*) or 40 mM K<sup>+</sup> (K<sup>+</sup>) and counted as in Fig. 2. Each experiment was done in duplicate, and the data are an average of 4 experiments. More than 800 neurons were counted in each condition. Error bars indicate  $\pm$  S.E.

with hCMV<sub>WT</sub> and those infected with hCMV<sub>m1-4</sub> in response to high K<sup>+</sup> or forskolin treatment (Fig. 6D).

The above results with the hCMV<sub>m1-4</sub> promoter suggest that either: (*a*) the CREs in the hCMV promoter are not involved in high K<sup>+</sup>-induced GFP expression, or (*b*) that the presence of the fifth, non-consensus, CRE in hCMV<sub>m1-4</sub> accounts for its inducibility. To decide between these possibilities, we built an adenovirus containing a construct with all 5 CREs disrupted (hCMV<sub>m1-5</sub>; Fig. 7A), and we assayed the mutated promoter's function in cultured hippocampal and SCG neurons. Fig. 7B shows hippocampal cultures infected overnight at day 8 *in vitro* with viruses containing hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub> and then treated for 24 h in control or high K<sup>+</sup> medium. Non-neuronal cells were GFP-positive indicating that the mutation of the CREs in the promoter does not affect this expression. Significantly, depolarizing cultures infected with hCMV<sub>m1-5</sub> constructs produced much less GFP expression in neurons compared with those infected with hCMV<sub>WT</sub> constructs (Fig. 7, *B* and *C*). We did similar experiments on cultured SCG neurons; our results indicated that mutating the 5 CREs largely inhibited induction both by high K<sup>+</sup> and by forskolin (Fig. 7D). To quantify hCMV<sub>m1-5</sub> promoter function further, we measured GFP mRNA levels with RNase protection assays from SCG neurons infected with either hCMV<sub>WT</sub> or hCMV<sub>m1-5</sub> and stimulated with high K<sup>+</sup> for 24 h (Fig. 8A). We observed that high K<sup>+</sup> induced a 94-fold increase in GFP mRNA from hCMV<sub>WT</sub> and only a 4.6-fold increase from hCMV<sub>m1-5</sub> (Fig. 8B). Therefore, disrupting the 5 CREs reduced high K<sup>+</sup> induction of GFP mRNA by over 95%; the mutations did not affect basal expression significantly. These results indicate that the high K<sup>+</sup> induction of the hCMV promoter is mediated through the 5 CREs and that *cis*-elements that bind other transcription factors contribute little to this effect.

Our results with the hCMV<sub>m1-5</sub>-GFP promoter/reporter construct demonstrate that the CREs in the hCMV promoter are essential for induction by depolarization in neurons. However, the difference in response to depolarization between hCMV<sub>m1-4</sub> and hCMV<sub>m1-5</sub> implies that either: (*a*) the fifth, non-consensus, CRE mediates the entire effect of depolarization from the wild-type hCMV promoter, or (*b*) the CREs in the promoter serve redundant functions in neurons and all five

must be removed to block depolarization-induced transcription. To resolve this, we made another construct in which we mutated only the fifth CRE ( $\text{hCMV}_{m5}$ ; Fig. 9A). We found that  $\text{hCMV}_{m5}$  responded to high  $K^+$  equally as well as the wild-type promoter (Fig. 9B), demonstrating that this fifth CRE is sufficient, but not necessary, for induction of the promoter by depolarization. This indicates that a functional redundancy exists among the five CREs.

#### DISCUSSION

The hCMV promoter is highly enriched with a densely packed array of *cis*-elements for a large number of transcription factors (3). Yet, in unstimulated neurons, activity from the hCMV promoter is weak; this suggests that the basal activities of many transcription factors are low in neurons, possibly to ensure a high signal-to-noise ratio for high fidelity regulation of target genes, an important consideration for activity-dependent neuronal plasticity. Moreover, our results suggest that in neurons the hCMV promoter acts as a molecular switch; in control, unstimulated neurons, transcription from the promoter is low, whereas in stimulated or electrically active neurons transcription from the hCMV promoter is high.

High  $K^+$  treatment strongly induces transcription from the hCMV promoter. Moreover, we find that CREB is phosphorylated by high  $K^+$  within 7.5 min and that phospho-CREB levels stay elevated for at least 24 h in high  $K^+$ . Nifedipine and KN-63 block both high  $K^+$ -induced CREB phosphorylation and hCMV promoter induction in sympathetic neurons, indicating that L-type  $\text{Ca}^{2+}$  channels and CaMK signaling are essential for both processes. Moreover, we show that activation of cAMP-dependent protein kinase with forskolin, which phosphorylates CREB directly, strongly induces expression from the hCMV promoter in neurons. These pharmacological data suggest that induction of hCMV promoter activity in neurons is regulated by a well defined pathway in which high  $K^+$  increases intracellular calcium, leading to the phosphorylation of CREB family members and induction of genes with CREs in their promoters (21). Interestingly, we found that the block of L-type  $\text{Ca}^{2+}$  channels was more effective in preventing high  $K^+$  induction of the hCMV promoter in sympathetic neurons than it was in hippocampal neurons. In hippocampal cultures, we observed that even in the presence of nifedipine, high  $K^+$  induced GFP expression in ~25% of the neurons. The likely reason is that high  $K^+$  evokes glutamate release from hippocampal neurons which acts on NMDA receptors and that the calcium influx through these receptors is sufficient to activate the hCMV promoter independent of L-type  $\text{Ca}^{2+}$  channels. Consistent with this, when we co-treated cultures with nifedipine and APV, an NMDA receptor antagonist, we blocked the high  $K^+$  induction completely (26). This suggests that for synaptically coupled hippocampal neurons, calcium influx through NMDA receptors is sufficient to activate the hCMV promoter. Therefore, it may be possible to use the hCMV-GFP promoter-reporter construct as a biosensor for monitoring activity-dependent gene expression in networks of living neurons over time.

To show directly that a CREB family member mediates the induced expression from the promoter, we mutated the CREs contained in the 19-bp repeats. Disrupting all five CREs ( $\text{hCMV}_{m1-5}$ ) blocks high  $K^+$ -induced expression in neurons by more than 95%. Basal activity of  $\text{hCMV}_{m1-5}$  is normal in non-neuronal cells indicating that these mutations specifically block hCMV promoter induction by stimuli that activate CREB in neurons. Interestingly, mutating the 4 consensus CREs alone ( $\text{hCMV}_{m1-4}$ ), or the fifth non-consensus CRE alone ( $\text{hCMV}_{m5}$ ), does not alter the induction by high  $K^+$ , indicating a functional redundancy among the 5 CREs in the native promoter. Whereas other studies suggest that the CREs in the

19-bp repeats enhance promoter activity in cell lines in response to elevated cAMP, their results differ from ours by suggesting that the effects of individual CREs are additive (9–11, 13). Differences in methodological approaches likely account for this discrepancy. The previous studies either used synthetic multi-19-bp repeat promoters or hCMV promoter deletion constructs in non-neuronal cell lines; in contrast, we specifically ablated the CREs in the promoter with site-directed mutagenesis. Our results demonstrate an essential role for the CREs in mediating a depolarization-dependent 90-fold increase in hCMV promoter activity in primary cultured neurons. Equally important, our results demonstrate that other *cis*-elements in the promoter that bind transcription factors thought to be regulated by activity-dependent mechanisms account for little, or none, of the increase in promoter function induced by depolarization.

Unlike most promoter studies, we used adenoviral mediated gene transfer to get our reporter construct into primary cultured neurons. Although the virus is replication-deficient, it is conceivable that there is an expression leak of adenoviral genes that may interact with the hCMV promoter complicating interpretation of these results. Consistent with our findings, it has been shown in primary cultured neurons infected with a replication-deficient adenovirus that reporter genes driven by hCMV are expressed at low levels; however, superinfection with replication-competent adenovirus, herpes simplex virus, or cytomegalovirus, which express downstream viral genes, increases expression of the reporter presumably by interaction of viral proteins with the hCMV promoter (6). To ensure that the response of the hCMV promoter to depolarization is not due to its adenoviral context and, furthermore, that it occurs in the context of chromatin, we have cultured sympathetic neurons from neonatal transgenic mice in which the hCMV promoter drives the expression of LacZ. In preliminary experiments, we observed that after 7 days in culture less than 1% of the neurons (2/236) expressed the transgene; however, in sister cultures stimulated with 40 mM  $K^+$  for 24 h over 60% (116/189) of the neurons contained  $\beta$ -galactosidase.<sup>2</sup> This result clearly demonstrates that induction of the hCMV promoter in neurons by depolarization is not dependent upon it being expressed in an adenoviral context.

The hCMV promoter is frequently used to express foreign genes. Our data indicate that transgene expression may be highly regulated in neurons by cellular events that alter the phosphorylation state of CREB. This complication is particularly relevant for interpreting studies on activity-dependent processes in nerve cells. A related concern is that the hCMV promoter is currently being used in studies aimed at developing gene therapies for treating neurological diseases (27, 28); since phospho-CREB levels are dynamically regulated in neurons, use of the hCMV promoter for gene therapy in the nervous system should be reconsidered.

Our work on the regulation of the hCMV promoter has implications for cytomegalovirus infections of the brain. In persons with AIDS, cytomegalovirus infections cause severe neurological abnormalities (1); moreover, congenital cytomegalovirus infections cause impaired brain growth, mental retardation, and progressive motor deficits in infants (2). Most healthy adults have been infected with cytomegalovirus, which remains in a latent, non-replicating form. A major field of study has been to identify the location of latent cytomegalovirus infections; much evidence suggests that a major site of latent infection is in CD34+ myeloid progenitor cells in the bone marrow (3). In-

<sup>2</sup> D. G. Wheeler, K. Hastings, and E. Cooper, unpublished observations.

triguingly, postmortem examinations of AIDS patients suggest that many neurons are latently infected with cytomegalovirus (1, 29, 30). Since activity of the hCMV promoter is rate-limiting for induction of productive cytomegalovirus infections (3), we suggest that neurons act as a reservoir for latent cytomegalovirus and that neuronal activity or other stimuli that activate CREB promote productive infections, initiating or accelerating the progression of CMV-related neurological disorders. Interestingly, herpes simplex virus type 1, a related virus, remains latent in sensory neurons and is re-activated from latency by activation of a CREB-dependent pathway (31).

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## REFERENCES

- Cohen, B. A., and Dix, R. D. (1997) in *AIDS and the Nervous System* (Berger, J. R., and Levy, R. M., eds) 2nd Ed., pp. 595–639, Lippincott-Raven Publishers, Philadelphia
- Whitley, R. J., and Stagno, S. (1997) in *Infections of the Central Nervous System* (Scheld, W. M., and Whitley, R. J., eds) 2nd Ed., pp. 223–253, Lippincott-Raven Publishers, Philadelphia
- Meier, J., and Stinski, M. F. (1996) *Intervirology* **39**, 331–342
- Mocarski, E. S., Kemble, G., Lyle, J., Greaves, R. F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11321–11326
- Boshart, M., Weber, F., Jahn, G., Dorsch-Häslner, K., Fleckenstein, B., and Schaffner, W. (1985) *Cell* **41**, 521–530
- Shering, A. F., Bain, D., Stewart, K., Epstein, A. L., Castro, M. G., Wilkinson, G. W., and Lowenstein, P. R. (1997) *J. Gen. Virol.* **78**, 445–459
- Fritschy, J.-M., Brandner, S., Aguzzi, A., Koedood, M., Lüscher, B., and Mitchell, P. J. (1996) *J. Neurosci.* **16**, 2275–2282
- Van den Pol, A. N., and Ghosh, P. K. (1998) *J. Neurosci.* **18**, 10640–10651
- Hunninghake, G. W., Monick, M. M., Liu, B., and Stinski, M. F. (1989) *J. Virol.* **63**, 3026–3033
- Fickenscher, H., Stamminger, T., Rüger, R., and Fleckenstein, B. (1989) *J. Gen. Virol.* **70**, 107–123
- Stamminger, T., Fickenscher, H., and Fleckenstein, B. (1990) *J. Gen. Virol.* **71**, 105–113
- Wilkinson, G. W. G., and Akrigg, A. (1992) *Nucleic Acids Res.* **20**, 2233–2239
- Rideg, K., Hirka, G., Prakash, K., Bushar, L. M., Nothias, J.-Y., Weinmann, R., Andrews, P. W., and Gönczöl, E. (1994) *Differentiation* **56**, 119–129
- De Koninck, P., and Cooper, E. (1995) *J. Neurosci.* **15**, 7966–7978
- Huetterer, J. E., and Baughman, R. W. (1986) *J. Neurosci.* **6**, 3044–3060
- He, T.-C., Zhou, S., Da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2509–2514
- Nyberg-Hoffman, C., Shabram, P., Li, W., Giroux, D., and Aguilar-Cordove, E. (1997) *Nat. Med.* **7**, 808–811
- Krieg, P. A., and Melton, D. A. (1987) *Methods Enzymol.* **155**, 397–415
- Mandelzys, A., Pié, B., Deneris, E., and Cooper, E. (1994) *J. Neurosci.* **14**, 2357–2364
- Le Gal La Salle, G., Robert, J. J., Berrard, S., Ridoux, V., Stratford-Perricaudet, L. D., Perricaudet, M., and Mallet, J. (1993) *Science* **259**, 988–990
- Shaywitz, A. J., and Greenberg, M. E. (1999) *Annu. Rev. Biochem.* **68**, 821–861
- Bito, H., Deisseroth, K., and Tsien, R. W. (1996) *Cell* **87**, 1203–1214
- Impey, S., Mark, M., Villacres, E., Poser, S., Chavkin, C., and Storm, D. R. (1996) *Neuron* **16**, 973–982
- Schorge, S., Gupta, S., Lin, Z., McEnery, M. W., and Lipscombe, D. (1999) *Nat. Neurosci.* **2**, 785–790
- Canaff, L., Bevan, S., Wheeler, D. G., Mouland, A. J., Rehfuss, R. P., White, J. H., and Hendy, G. N. (1998) *Endocrinology* **139**, 1184–1196
- Wheeler, D. G., and Cooper, E. (2000) *30th Annual Society for Neuroscience Meeting*, Nov. 4–9, New Orleans, Abstr. 613.6
- Kordower, J. H., Emborg, M. E., Bloch, J., Ma, S. Y., Chu, Y., Leventhal, L., McBride, J., Chen, E.-Y., Palfi, S., Roitberg, B. Z., Brown, W. D., Holden, J. E., Pyzalski, R., Taylor, M. D., Carvey, P., Ling, Z. D., Troner, D., Hantraye, P., Déglon, N., and Aebsicher, P. (2000) *Science* **290**, 767–772
- Thomas, C. E., Schiedner, G., Kochanek, S., Castro, M. G., and Löwenstein, P. R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7482–7487
- Wiley, C. A., Schrier, R. D., Denaro, F. J., Nelson, J. A., Lampert, P. W., and Oldstone, B. A. (1986) *J. Neuropathol. Exp. Neurol.* **45**, 127–139
- Clifford, D. B. (1999) *Semin. Neurol.* **19**, 185–192
- Millhouse, S., and Wigdahl, B. (2000) *J. Neurovirol.* **6**, 6–24