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Glial- and Fat-Specific Expression of the Rat Glycerol Phosphate Dehydrogenase-Luciferase Fusion Gene in Transgenic Mice

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Glycerol phosphate dehydrogenase (GPDH) is a metabolic enzyme that catalyzes the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate. It provides phospholipid precursors for lipid biosynthesis and energy metabolism. In the brain, GPDH enzymatic activity, protein, mRNA are exclusively associated with oligodendroglial and Bergmann glial cells. Expression of GPDH in the brain increases dramatically during the active period of myelination, and is regulated by extracellular signals. In an effort to understand the mechanism that confers glial-specific expression of GPDH, we have examined the role of the 5′ flanking sequence of the rat GPDH gene in conferring cell-specific expression of reporter gene in transgenic mice. Luciferase reporter constructs containing either the full-length GPDH 5′ flanking region (p4.3), or a distally truncated version (p2.6), were injected into mouse zygotes. Three independent lines of transgenic mice containing the p4.3, and seven lines of mice containing the p2.6 constructs, were analyzed. Luciferase enzyme activity was detectable only in brain and fat, not in other GPDH-positive organs such as liver, muscle, and kidney. Both the full-length and the distally deleted transgenes were expressed similarly in these two organs, indicating that the distal portion of the 5′ flanking region was not required for brain- and fat-specific expression. Immunocytochemical analyses revealed that luciferase immunoreactivity colocalized with glial fibrillary acidic protein (GFAP)-positive Bergmann glia in the cerebellum, and myelin basic protein (MBP)-positive oligodendroglia in the cerebral cortex and the brainstem. Results here suggest that the rat GPDH 5′ flanking region directs glial-specific expression of GPDH transcription in the brain, and provide a good model for analyses of changes in glial metabolism in response to extracellular perturbations in vivo. J. Neurosci. Res. 50:300–311, 1997. © 1997 Wiley-Liss, Inc.

Key words: glycerol phosphate dehydrogenase; transgenic mice; oligodendrocytes; Bergmann glia; tissue-specific expression; luciferase

INTRODUCTION

The metabolic enzyme glycerol phosphate dehydrogenase (GPDH; E.C.1.1.1.8) catalyzes the conversion of dihydroxyacetone phosphate to glycerol phosphate, a precursor for triglyceride synthesis. In the brain, GPDH protein and mRNA levels are exclusively enriched in oligodendroglia (deVellis et al., 1977; Leveille et al., 1980; Kumar et al., 1986; Gordon et al., 1992; Fisher et al., 1981) and Bergmann glial cells (Fisher et al., 1981). Expression of GPDH in these cells is subject to developmental, genetic, and epigenetic factor regulation (for review see Kumar and de Vellis, 1988). For example, GPDH mRNA level dramatically increases during the second postnatal week of development, which corresponds to the active period of myelination (Laatsch, 1962; Kumar et al., 1985). GPDH expression in oligodendroglial cells is also up-regulated by the steroid hormone glucocorticoids (Kumar et al., 1989). The glucocorticoid induction has been recently correlated to be to a stress-inducible event in the brain (Finch et al., 1990; Nichols et al., 1996), indicating that GPDH is part of the changes in glial metabolism in response to stress or increase in glucocorticoids. Additionally, GPDH expression in Bergmann glia is also dependent on appropriate contact with neighboring Purkinje cells, and thyroid hormone level (Fisher, 1984). These evidence suggest that GPDH expression in glial cells may be an indicator of changes of glial cell metabolism in response to extracellular stimuli. Since many of the changes in GPDH enzymatic activity and protein level have been documented at the message accumulation level, or transcription run-off rate level (Ratner et al., 1981; Kumar and de Vellis, 1988; Kumar et al., 1986, 1989, 1990; Cook et al., 1985; Prochazka et al., 1989), it is likely that transcription of GPDH is highly regulated, and that a complex array of regulatory elements exist to mediate regulation of GPDH transcription.

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Previously, the 5′ flanking regions of the mouse and the human GPDH genes were isolated and determined (Djian et al., 1985; Phillips et al., 1986; Ireland et al., 1986; Gwynn et al., 1990). Sequence comparison revealed that the 5′ flanking region of the GPDH-encoding gene was highly conserved (Gwynn et al., 1990). Regions of high conservation termed the ''distal promoter region'' (DPR) and the ''proximal promoter region'' (PPR), were identified. Several DNaseI hypersensitive sites were also found in the 5′ flanking region of the human GPDH gene (Gwynn et al., 1990). These data indicate that the 5′ flanking region of GPDH contains conserved sequences that bind to transcription factors, and may be important for regulation of GPDH transcription.

In addition to the sequence analyses, the function of the genomic structure of GPDH gene has also been tested in transgenic mice. Transgenes containing the entire genomic structure, including the 5′ flanking region, intron/exons, and the 3′ flanking region, were found to be expressed tissue-specifically in transgenic mice (Kozak et al., 1991; Birkenmeier et al., 1992). However, the elements important for glial cell-specific expression of GPDH remain elusive. Additionally, the role of the conserved 5′ flanking sequence alone in conferring tissue- and cell-specific expression of GPDH has not been addressed. Here we extend the study of previous transgenic experiments by generating transgenic mice containing the 5′ flanking region of the rat GPDH gene alone in front of the luciferase reporter gene. The ability of the rat GPDH 5′ flanking region to direct the luciferase reporter gene tissue- and cell-specifically was indirectly assessed by enzymatic and immunocytochemical methods. Results here show that luciferase enzymatic activities were only detectable in fat and brain, not in other GPDH-producing organs. Immunocytochemical analyses of transgenic mouse brain revealed that luciferase immunoreactivity was localized to the Bergmann glia of the cerebellum, and to the oligodendroglia of the cerebral cortex and brainstem. The glial-specific expression of the transgene defines a functional role for the 5′ flanking region of the GPDH gene in regulating GPDH transcription. The transgenic mice established here also provide a useful model to study environmental regulation of glial cells in vivo.

MATERIALS AND METHODS

DNA Used for Generation of Transgenic Mice

Two different lengths of the GPDH 5′ flanking region were used to produce reporter constructs for transgenic mice: a full-length construct, p4.3; and a truncated version, p2.6 (Fig. 1). The plasmid p4.3 contains a PstI-NcoI restriction fragment spanning 4,230 basepairs of the rat GPDH 5′ flanking sequence from a genomic clone of the rat GPDH DNA (Cheng and de Vellis, submitted). This includes sequences upstream of the ATG start codon of GPDH exon 1, the presumed transcription initiation site, and 409 basepairs of sequences from D15kz (the transcriptional unit located upstream of GPDH; Johnston et al., 1989). The plasmid p2.6 contains a BstXI-NcoI restriction fragment spanning 2,456 basepairs of the 5′ flanking region. This construct was deleted at the distal portion, and thus did not include sequences from the upstream gene D15kz1, and 1,356 basepairs of the distal portion of GPDH 5′ flanking sequences. Both fragments were blunt-end-cloned into the XhoI site upstream of the luciferase reporter gene in the expression vector pGL2-Basic (Promega, Madison, WI). To prepare the construct DNA for injection, both plasmids were purified in large scale using Qiagen Maxi-prep columns. Purified plasmid DNA was digested with KpnI and BamHI to eliminate the vector sequences, electrophoresed, gel-purified, ethanol precipitated, and resuspended in 1X TE. The resulting purified DNA fragment, including the luciferase cDNA, was 6,956 basepairs long for p4.3, and 5,182 basepairs long for p2.6 (Fig. 1).

Transgenic Mice

Microinjection of DNA into the pronucleus of zygotes was performed by the Transgenic Mouse Facility at UCLA. The founder mice were created using the strain (C56BL/6J × C3H). Positive transgenic animals (F0) were mated to their negative siblings to produce F1 offspring. Each transgenic line was then established and maintained by brother-sister mating of positive mice. All mice were housed at the UCLA Laboratory of Biomedical and Environmental Sciences vivarium facility and maintained under strict light cycles from 0600 to 1800.

Screening of Transgenic Mice

A piece of tail (1 cm) was excised from ear-marked mice and incubated with proteinase K (100 μg/ml) in 50 mM tris, 0.5% SDS, and 100 mM EDTA at 55°C overnight. One round of phenol and one round of phenol/chloroform extraction were performed before the DNA was precipitated. After precipitation, the DNA was resuspended in tris EDTA(TE) and the concentration was determined in a spectrophotometer. Ten micrograms of the genomic DNA were digested with EcoRI, electrophoresed on 0.8% agarose gels, and transferred to nylon membranes. The nylon membranes were incubated with randomly primed luciferase cDNA sequence (a 616-bp HindIII-EcoRI fragment from pGL2-Basic) using standard hybridization conditions of 6× SSC at 42°C (Sambrook et al., 1989). After overnight hybridization, the membranes were washed in progressively stringent condi-

tion to a concentration of 0.1× SSC at 37°C. The blots were exposed to X-ray film between 1 and 7 days and developed.

Preparation of Tissue Extracts

Tissue samples were dissected from CO_2-euthanized mice and placed in 10 volumes of ice-cold harvest buffer (50 mM NaH_2PO_4, pH 7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% glycerol). Brains were dissected into three regions: the cerebral cortex, the cerebellum, and the brainstem. Homogenization of brain regions was performed using a teflon-glass Potter-Elvehjem homogenizer. Peripheral tissues such as muscle, liver, kidney, and spleen were homogenized with a blade homogenizer. Homogenates were centrifuged at 10,000 $\times$ *g* in a refrigerated microfuge for 10 min. The supernatant was transferred to a fresh tube and used in subsequent enzymatic analysis. Control experiments have been performed to ensure that the different homogenization methods used between brain and peripheral tissues did not produce differential preservation of GPDH and luciferase enzymatic activities.

Assay of GPDH and Luciferase Activities

Tissue homogenates were diluted 1:5 with harvest buffer before assay. GPDH assay was performed according to that previously described (Kumar et al., 1989). Briefly, 100 ml of the diluted tissue homogenates were added to 800 ml of 0.166 mM nicotinamide adenine dinucleotide-reduced (NADH). The reaction was initiated by addition of 100 ml of 3.3 mg/ml dihydroxyacetone phosphate. Decreases in optical absorbance of NADH at wavelength 340 nm were recorded in the spectrophotometer. GPDH enzymatic activity was calculated based on nanomoles of NADH hydrolyzed per minute per milligram protein.

Luciferase assays were performed using a luminometer. One hundred microliters of the luciferase reaction buffer (530 µM ATP, 470 µM luciferin, 270 µM coenzyme A, 20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 1.07 mM $(MgCo_3)_4Mg(OH)_25H_2O$ and 33.3 mM DTT) was injected into 30 ml of tissue homogenate in a tube placed inside of a luminometer. Light emission was measured for 10 seconds after addition of the reagent, and results were expressed as light units. Subsequent to GPDH and luciferase assay, protein content was determined using the standard Bradford method.

Immunohistochemistry

Mice were anesthetized with ether and perfused intracardially with saline (0.9% NaCl) followed by 3.7% formaldehyde in phosphate-buffered saline (PBS). Brains were dissected and immersed in the formaldehyde fixative overnight at 4°C, followed by 30% sucrose in PBS for an additional day at 4°C. Cryoprotected brains were then embedded in the OCT polymer and stored at −20°C until cryosectioning. Frozen embedded brains were sectioned sagittally at 10- to 12-µm thickness, transferred to slides, dried for 15 to 30 min at 40°C, and stored at −20°C until immunostaining. For immunostaining, sections were rinsed in PBS and permeabilized with 1% Triton X-100 for 20 min at 37°C and 10 min at room temperature. After rinsing with PBS, the permealized sections were incubated with blocking solution (5% normal goat serum in PBS and 0.1% Triton X-100) at 37°C for an hour. The sections were then labeled with primary antibodies (at 1:100 dilution for luciferase, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP, Espinosa and de Vellis, 1988), 1:1,000 dilution for the GPDH antibodies) at 4°C overnight. Primary antibody against beetle luciferase was purchased from Promega; antibody against purified rat GPDH was generated in this laboratory (Kumar et al., 1985). Following incubation, the sections were washed three times with tris-buffered saline (TBS), and incubated with fluorescein- (for GFAP or MBP antibodies) or Texas red-conjugated (for luciferase and GPDH) secondary antibodies at room temperature for 1 hour. After extensive washing with TBS, the sections were mounted on coverslips with Vinol, and observed and photographed using a fluorescent microscope.

RESULTS

GPDH-Luciferase Transgenic Mice

The transgenic mouse model was developed to determine if the 5′ flanking region of the rat GPDH gene directs tissue- and cell-specific endogenous GPDH transcription. Two different lengths of the 5′ flanking region were tested in transgenic mice: a 4.3-kb construct (p4.3) containing all of the GPDH 5′ flanking sequence, including 409 basepairs of sequences from the upstream gene D15kzl; and a 2.6-kb construct (p2.6) containing 2,456 basepairs of the 5′ sequence. The p2.6 construct is devoid of the sequence from the upstream gene, and contains all but 100 basepairs of the conserved DPR and all of the PPR (Fig. 1). The TATA-like box, the conserved transcription initiation site, and all of the 5′ untranslated region of the endogenous GPDH gene up to the first ATG were all included in both constructs. Downstream of the luciferase cDNA, an SV40 polyadenylation signal and splice donor/acceptor sequences were also included to facilitate transcriptional termination and splicing. The length of the transgene, from the beginning of the GPDH 5′ flanking region, to the end of the SV40 polyadenylation/splice signal downstream of the luciferase cDNA, is 6,956

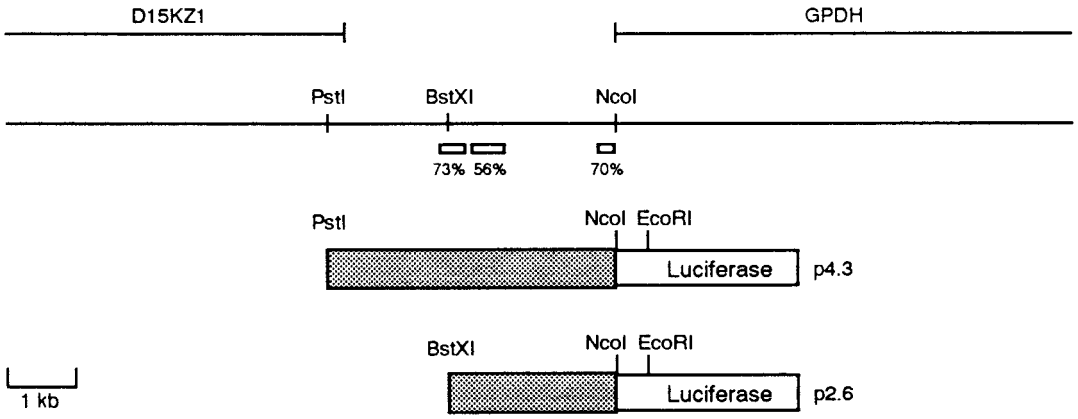

Fig. 1. Structure of the transgenes containing the rat GPDH 5′ flanking region fused in front of the luciferase cDNA. The thick lines on top labeled GPDH and D15kz1 represent the transcribed regions of the GPDH and D15kz1 genes, respectively. D15kz1 is a transcriptional unit located approximately 3.8 kb upstream of GPDH exon 1 that encodes a message with no homology to any known genes or proteins. The small boxes underneath the thick lines represent regions highly conserved between the mouse and the human GPDH 5′ flanking sequence, with the percent conservation noted under each box. The restriction sites NcoI, BxtXI, and PstI were used to excise the 5′ flanking sequence out and clone into the luciferase constructs. The p4.3 construct contained the PstI-NcoI fragment, whereas the p2.6 construct contained BxtXI-NcoI fragment. A unique EcoRI site exists within the luciferase cDNA. This site was used for diagnosis purpose of transgene integrity and arrangement in Southern blots of genomic DNA.

basepairs long for construct p4.3, and 5,182 basepairs for construct p2.6.

Purified fragments of DNA were microinjected into fertilized mouse eggs; 113 animals were born and screened for the presence of a transgene by Southern blot analysis of genomic DNA. Identification of positive mice was based on hybridization to a 600-bp HindIII-EcoRI luciferase cDNA fragment. Positive mice containing the p4.3 transgene were named C57BL/C3H-TgN(GPDluc)43JdV, and those containing p2.6 were named C57BL/C3H-TgN(GPDluc)26JdV according to the Rules and Guidelines for Transgenic Mice (Davisson, 1994). However, in the remainder of the text, they will be abbreviated as Tg4.3 and Tg2.6, respectively.

Screening of the Transgenic Founders

The enzyme EcoRI was chosen to digest the mouse genomic DNA, because it has a unique restriction site within the entire length of the transgene (Fig. 1). EcoRI-digested genomic DNA containing an intact transgene should have a minimal length corresponding to the length of the transgene from its distal end to the EcoRI site. Accordingly, all founders from Tg4.3 g gave signals higher than 4,860 basepairs, and all except one founder from Tg2.6 (founder 48) gave signals higher than 3,086 basepairs (Fig. 2). Because the sizes of the hybridized signals were all larger than the minimal predicted lengths, these founders most likely contained transgenes that were intact at the 5′ portion. Founder 48 of Tg2.6 contained a hybridization signal at 2 kb, indicating that the transgene was truncated at the 5′ end before integration. However, founder 48 also contained two less intense bands at 3 and 5 kb, suggesting that at least one intact copy of the transgene was present in the genome.

The unique EcoRI site also revealed that most of the founders contained multiple copies of the transgene arranged in tandem array. Because EcoRI cleaves only once in the transgene, multiple copies of the transgenes that integrated in a head-to-tail array will yield a hybridization signal whose size is identical to the size of the transgene. Examination of the hybridization signals on Southern blots revealed that all Tg4.3 founders contained a 7-kb band (Fig. 2A), indicating all three founders of Tg4.3 contained multiple intact copies of the transgene arranged in a tandem array. On the other hand, hybridization of Tg2.6 appeared more heterogenous. Four out of eight founders from Tg2.6 had an intense hybridization signal at 5,182 basepairs (founders 4, 17, 37, and 59; Fig. 2B), which corresponded to the length of p2.6. Founders 13, 17, 21, and 28 had signals at other locations, suggesting that they either contained single copies, or multiple copies of the transgenes in nontandem arrangements.

All founders were fertile, and transmitted the transgenes to their offspring. Founder 28 died of unknown reasons soon after tail biopsy, so it was not included in the analysis. Therefore, three lines of Tg4.3, and seven lines of Tg2.6 were propagated and analyzed. The hybridization pattern of all offspring DNAs were similar to their founders', indicating that the transgene was transmissible and stable in the genome.

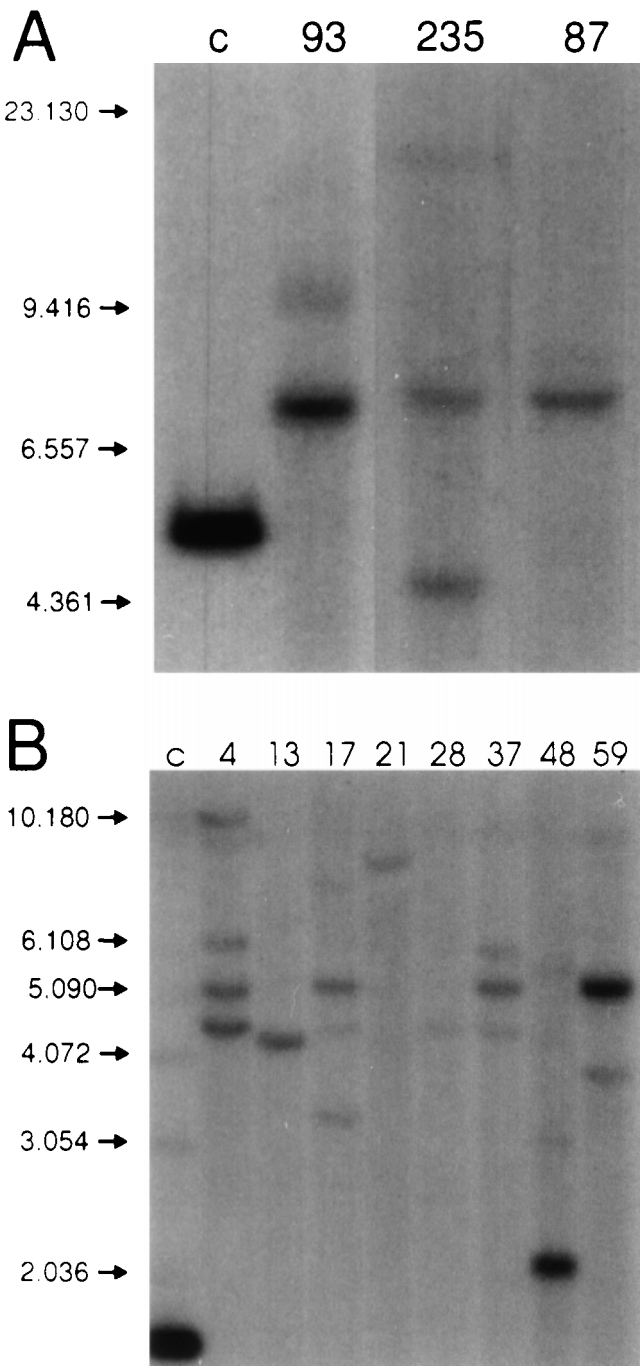

Fig. 2. Southern analysis of transgenic founder genomic DNA hybridized to the luciferase cDNA probe. **A:** Hybridization pattern of Tg4.3 mouse DNA. **B:** Hybridization of Tg2.6 mouse DNA. c, control DNA that contains ten copies of the luciferase cDNA. The numbers on top of each lane represents the founder number. Numbers next to the arrows represent the sizes of molecular weight markers on the gel. Ten micrograms of genomic DNA (10 μg) were digested with EcoRI, fractionated on 0.8% agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled luciferase cDNA (a 616-bp HindIII-EcoRI fragment from pGL2-Basic). Blots were exposed to X-ray film for 3 days.

Endogenous GPDH Activity in Transgenic Mice

GPDH activity in various tissues of the mouse has been extensively characterized (Finch et al., 1990; Fisher, 1984). Activity levels of GPDH in adult mice are known to vary two orders of magnitude from a low of 50 unit/mg in the spleen, to a high of 3,000 units/mg in the brown fat (Ratner et al., 1981). More importantly, it has been established that GPDH enzymatic activity is proportional to the amount of immunoreactive GPDH protein as well as to the amount of translatable GPDH mRNA (Ratner et al., 1981). This allowed us to use the level of GPDH enzymatic activity as an indicator of the level of endogenous GPDH gene transcription.

Five different tissues, including brain, muscle, liver, spleen, and fat, were analyzed for GPDH activity (Fig. 3). Because of our particular interest in GPDH expression within the brain, the brain was further dissected into three regions: the cerebral cortex (cx), the brainstem (bs), and the cerebellum (cb). Among all the tissues examined, GPDH activity was the highest in fat. Muscle, liver, and kidney each expressed a moderate amount of GPDH activity, while the spleen GPDH expression was barely detectable. Within the brain, cerebellar GPDH activity was approximately 2-fold more than cerebral cortex and brainstem. No difference in GPDH activity was observed between transgenic and wild-type mice (Fig. 3), except in the muscle tissues of Tg4.3. This indicates that the presence of the transgenes had little effect on endogenous GPDH expression.

Transgene Expression Is Brain- and Fat-Specific

To assess the transcriptional activity of the GPDH 5′ flanking region, luciferase enzymatic activity was measured. The same tissue homogenates used to assay GPDH activity were assayed for luciferase activity. The composition of the assay buffer was empirically determined such that activities of both pure enzymes were preserved. Luciferase activity, expressed in light units per mg protein, was detected only in brain and fat tissues of most transgenic mice (Fig. 4). Values of luciferase activities in other tissues were close to background (0.1 light unit/mg protein). A few exceptions exist; for example, one line of Tg4.3 (Fig. 4, line 93) expressed a moderate amount of luciferase activity in the kidney, while two other lines (Fig. 4, 87 and 235) had no detectable luciferase activity in the kidney. In Tg4.3 mice (Fig. 4A, lines 87, 93, and 235), luciferase activity was consistently higher in the cerebral cortex than in the brain stem or the cerebellum. However, in Tg2.6 mice (Fig. 4B), the levels of luciferase activities in different brain regions appeared to be more variable. Qualitatively, luciferase activity was consistently expressed in the brain and in fat. Other organs, such as liver, muscle, kidney, and spleen had no appreciable luciferase expression.

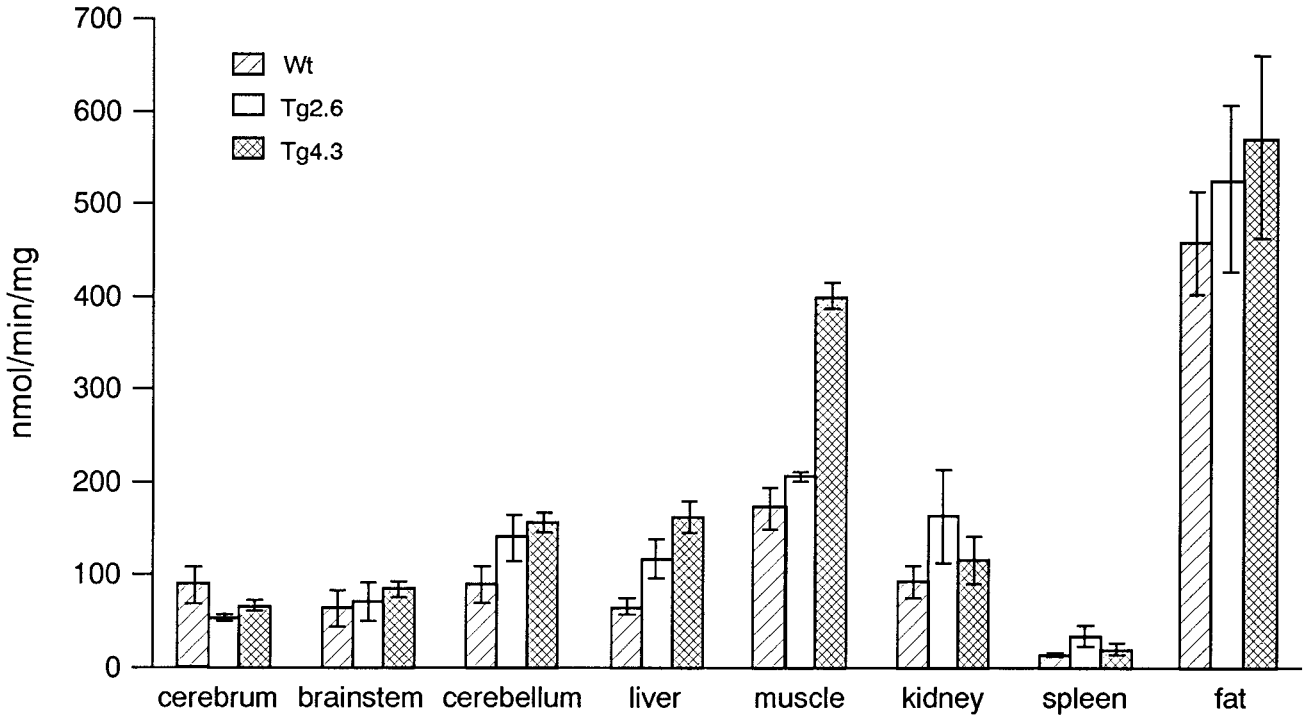

Fig. 3. Distribution of the endogenous GPDH enzymatic activity in cerebrum, cerebellum, brainstem, liver, muscle, kidney, spleen, and fat of wild type and transgenic mice. GPDH activity was determined from a 10,000 × *g* supernatant of tissue homogenates, and is expressed in nanomoles of NADH hydrolyzed per minute per milligram total protein. The results are an average of three independent experiments with triplicate samples in each experiment.

Two lines of Tg2.6 (Fig. 4, lines 13 and 37) had no detectable luciferase activities in all tissues examined, as values of light units were indistinguishable from the background. To ensure that the lack of luciferase expression in those two lines was not due to a high rate of luciferase degradation, homogenates from the brains of Tg4.3-positive mice were mixed with the homogenates from Tg2.6-negative mice in a 1:1 ratio. Luciferase activity of the positive homogenate was not affected by the presence of negative tissue homogenates. A similar mixing experiment was also performed for homogenates of liver, muscle, kidney, and spleen. No significant decrease in luciferase activity was detected when the homogenates of these tissues were mixed with the luciferase-expressing homogenates. Therefore, the lack of luciferase activity in these tissues most likely was not due to the existence of a more active degradation mechanism for the luciferase enzyme, but rather, to a lower production of the enzyme.

Localization of Luciferase-Expressing Cells in the Brain

GPDH expression in the mouse and the rat cerebellum has been previously characterized (de Vellis et al., 1977; Leveille et al., 1980; Gordon et al., 1992; Fisher et al., 1981). Antibodies against the adult isozyme of mouse GPDH (Fisher et al., 1981), or antiserum against the purified rat brain GPDH enzyme (Leveille et al., 1980) consistently labeled oligodendroglia and Bergmann glia in the mouse cerebellum. Neurons and astroglial cells, on the other hand, do not express GPDH at levels detectable by immunocytochemical procedure. The exclusive presence of GPDH immunoreactivity in glial cells has made GPDH an immunocytochemical marker for glial cells in vivo, and was used in this study to correlate the luciferase-expressing cells with GPDH-positive cells. However, double-immunolabeling using these two antisera cannot be performed on the same section, because the luciferase antiserum was derived from the same host (rabbit) as the GPDH antiserum. Therefore, luciferase and GPDH staining was performed individually on independent slides. Patterns of immunostaining from each of these two preparations were then compared.

In the cerebellum of the transgenic mouse, GPDH immunoreactivity was detected in cell body-like structures beneath the Purkinje cell layer (p; Fig. 5A). In addition, immunoreactive fibers radially oriented from the Purkinje layer to the pial surface were also prominent (arrowheads, Fig. 5A). The pattern of staining characteristic of the Berg-

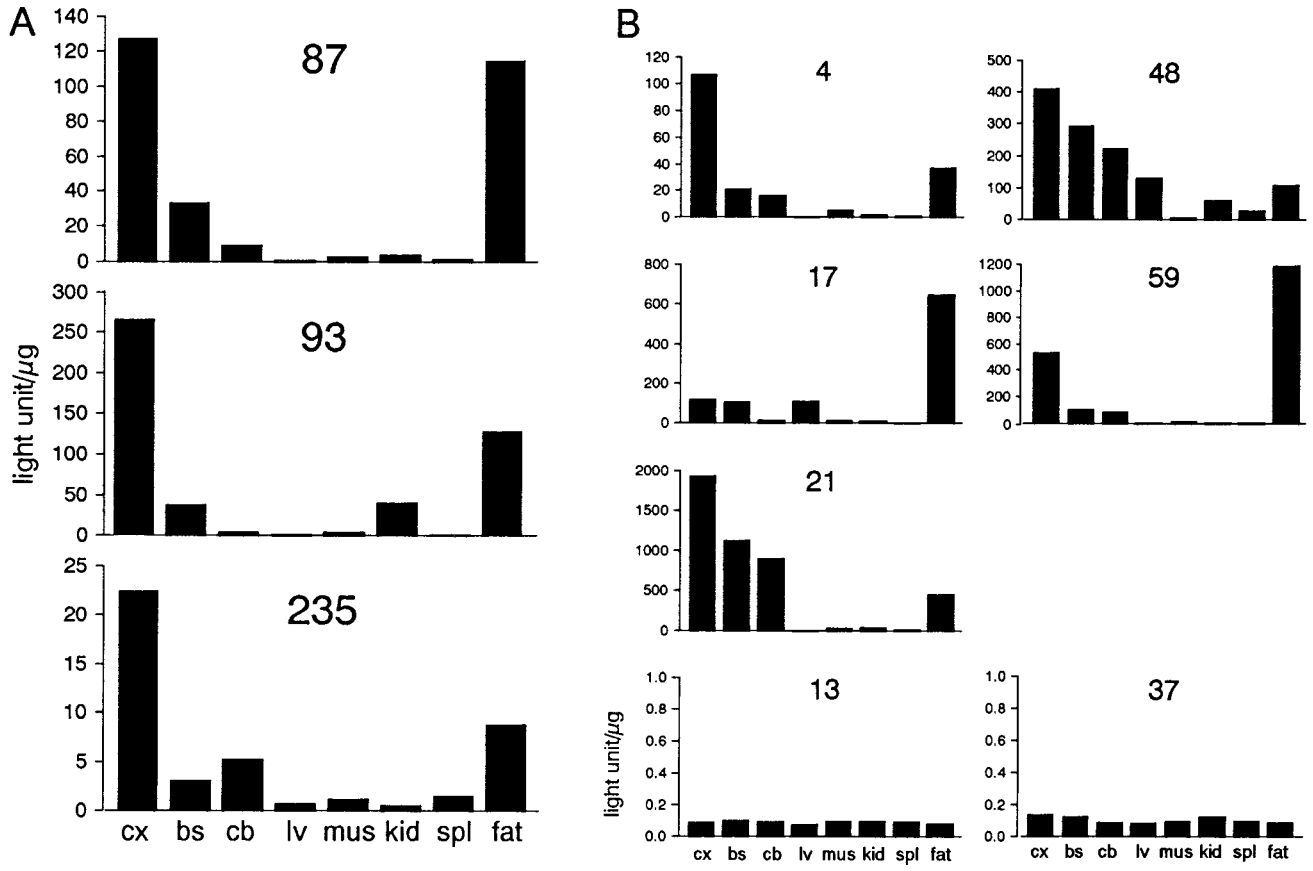

Fig. 4. Distribution of luciferase activity in each line of GPDH-luc transgenic mice. **A:** Expression pattern of three lines of Tg4.3 **B:** Expression pattern of seven lines of Tg2.6 mice. The number on top of each graph represents the number of the transgenic line. cx, cerebral cortex; bs, brainstem; cb, cerebellum; lv, liver; mus, muscle; kid, kidney; spl, spleen. Luciferase activity was expressed as light unit per microgram protein. At least three animals were used for each determination. The results are an average of three independent assays with triplicate samples for each assay. All standard deviations are less than 10%.

mann glial cells and fibers (Palay and Chan-Palay, 1974) is also very similar to the GPDH staining patterns obtained by Fisher et al. (1981) in the mouse cerebellum. A small number of positively stained cells, corresponding to oligodendroglia, as are also found scattered throughout the granular layer and white matter. The same staining pattern was also observed in equivalent sections incubated with the preimmune control serum (data not shown).

Similar to the GPDH staining pattern, luciferase immunoreactivity was also prominent in the cell body-like structures beneath the Purkinje layer (Fig. 5B). Although no prominent immunoreactive fibers were detectable at the molecular layer, small punctate staining was scattered throughout this region (arrows, Fig. 5B), at locations similar to the GPDH-immunoreactive fibers. However, unlike GPDH staining, no distinctive luciferase immunoreactivity was detectable in the white matter of the cerebellum. The staining was specific, as no luciferase-immunoreactive cells were detectable in equivalent sections of nontransgenic littermates (Fig. 5C).

The similarity in position and morphology between GPDH- and luciferase-immunoreactive cells suggested that luciferase was expressed in GPDH-producing Bergmann glial cells in the cerebellum. To further confirm the identity of luciferase-positive cells, we double-immunolabeled the transgenic sections with the luciferase antiserum and the antibody against GFAP. GFAP is an intermediate filament protein that specifically labels astroglial cells and Bergmann glial cells (Bignami and Dahl, 1974; Bignami et al., 1972). A colocalization of luciferase immunoreactivity with GFAP would further support the identity of these cells as Bergmann glia in the cerebellum. Figure 5D demonstrates that the GFAP-immunoreactive cell bodies (Fig. 5D) were also found beneath the Purkinje cell layer, with radial fibers extending towards the pial surface. Correspondingly, these GFAP-positive

cells were also luciferase-positive (Fig. 5E), further supporting that these cells are Bergmann glial cells.

Outside of the cerebellum, luciferase staining appeared more dispersed. No intense staining, comparable to the layer of staining at the Purkinje layer in cerebellum, was observed (Fig. 6A, C, E). Instead, small punctate staining was visible in clusters in the cerebral cortex and the brainstem. Interestingly, luciferase-positive cells in these two brain regions did not colocalize with GFAP immunoreactivity (data not shown). In an effort to identify the cell type that expresses luciferase in the cerebral cortex and the brainstem, we double-immunolabeled transgenic sections with the luciferase antiserum and an oligodendrocyte-specific antibody against MBP. Under low magnification (200×; Fig. 6B), MBP immunoreactivity was prominent in the white matter tract area, but also visible in areas outside of the white matter tract. On the other hand, luciferase immunoreactivity in the same section (Fig. 6A) was absent in the white matter tract, but prominent in areas outside of the white matter tract. Examination of this area outside of the white matter tract under higher magnification (400×) revealed that luciferase immunoreactivity colocalized with MBP immunoreactivity (Fig. 6C and D). A similar colocalization of the luciferase and MBP immunostaining was also observed in the brainstem (Fig. 6E and F), indicating that luciferase-expressing cells in the cerebral cortex and the brainstem correspond to MBP-positive oligodendroglial cells. The staining patterns presented in Figures 5 and 6 were observed from brain sections of Tg2.6 line 59 mice. Similar results were also obtained using brain sections from Tg4.3 line 93, and Tg2.6 line 21 (data not shown).

DISCUSSION

In this study, we tested the transcriptional activity of the 5′ flanking region of the rat GPDH gene in transgenic mice. Two different lengths of the 5′ flanking region, namely a 4.3-kb and a 2.6-kb fragment, were tested in luciferase reporter constructs. The p4.3 construct contained all of GPDH 5′ flanking region, from the first ATG codon of exon 1 upstream to the PstI site within the upstream gene D15kz1. The p2.6 construct contained a 2.5-kb 5′ flanking sequence, which included most of the ''Distal Promoter Region'' and all of the ''Proximal Promoter Region'' known to be conserved between the mouse and the human GPDH 5′ flanking sequences (Fig. 1; Gwynn et al., 1990). Luciferase assays showed that all of the Tg4.3 lines, and five out of seven Tg2.6 lines expressed the transgene in the brain and in the fat. Luciferase activity was negligible in other GPDH-positive organs, such as muscle, liver, and kidney. Additionally, no luciferase activity was detectable in the GPDH-negative spleen. Taken together, these results indicate that the 5′ flanking region of the GPDH gene alone was sufficient to confer fat- and brain-specific transcription, and that the distal 1.8-kb of deleted sequence, containing most of the nonconserved sequence between mice and humans, was not required for GPDH transcription in the brain and in the fat.

In two previous studies, the entire genomic structure of either the mouse or the human GPDH gene, including the 5′ flanking region, intron/exons, and the 3′ flanking region, was tested in transgenic mice (Kozak et al., 1991; Birkenmeier et al., 1992). Transgenes in these configurations were found to be expressed in all GPDH-positive tissues, including brain, fat, kidney, liver, and muscle (Ireland et al., 1986). The fact that the transgenes containing the 5′ flanking region alone were expressed in the brain and the fat indicates that the fat- and the brain-specific enhancer elements are located in the 5′ flanking region, whereas the elements necessary for expression in muscle, kidney, and liver reside in the sequence downstream of exon 1, most likely within the introns and the 3′ flanking sequences.

In addition to enzymatic assays, we also used immunocytochemical methods to determine the identity of luciferase-expressing cells in the brain. Results revealed that the morphology and the position of luciferase-immunoreactive cells highly resemble those of the GPDH-immunoreactive cells. The cell body-like structures in the Purkinje layer corresponded to the location of Bergmann glial cells in the cerebellum. Although luciferase-immunoreactive fibers in the molecular layer were not as prominent as the GPDH-immunoreactive fibers, distinct punctate staining can be seen scattered throughout this region. This difference in the morphology of the immunoreactive fibers between GPDH and luciferase most likely is a result of differential subcellular localization between the two proteins. The luciferase protein is known to be targeted to peroxisomes (Keller et al., 1987), while GPDH is distributed throughout the cytoplasm. The fact that the pattern of GPDH staining appeared more diffuse, whereas luciferase staining appeared smaller and more punctate, suggests that they may have differentially localized in different compartments inside the cell. Nevertheless, luciferase immunoreactivity colocalized with GFAP immunoreactivity in the cerebellum, supporting that luciferase was expressed in Bergmann glial cells of the transgenic cerebellum.

Outside of the cerebellum, luciferase immunoreactivity was not associated with GFAP. Instead, clusters of small punctate luciferase staining colocalized with MBP immunoreactivity. Since MBP is specifically associated with myelin, the colocalization of MBP and luciferase immunoreactivity indicates that the luciferase-expressing cells in the cerebral cortex and the brainstem are oligodendroglial cells. Although most of the luciferase immunore-

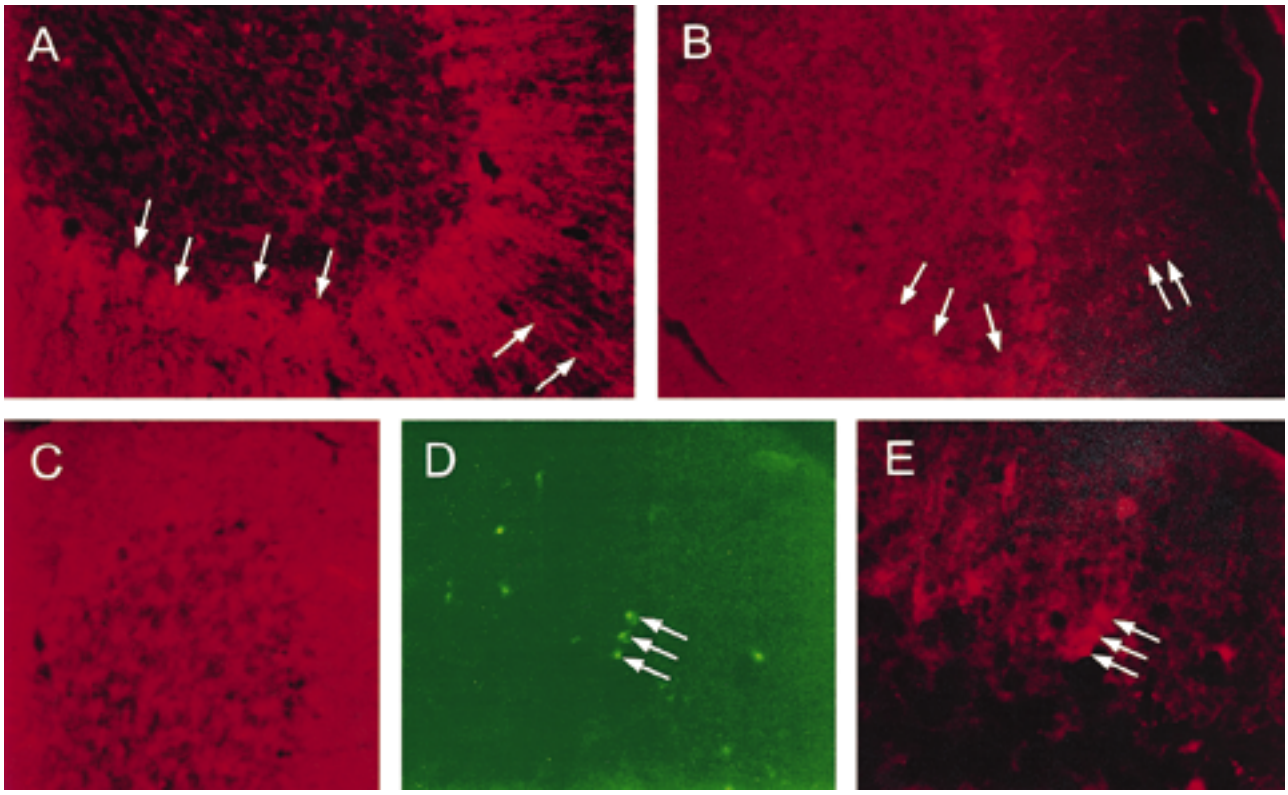

Fig. 5. Immunocytochemical localization of luciferase-expressing cells in the cerebellum of transgenic and control mice. **A:** GPDH immunofluorescence. **B:** Luciferase immunofluorescence in transgenic mouse cerebellum. Magnification, 200×. Arrows, immunoreactive fibers in the molecular layer. **C:** Luciferase immunofluorescence in the cerebellum from a nontransgenic littermate. The fluorescence signals were visualized by UV microscopy using a rhodamine filter. **D:** GFAP immunostaining of transgenic cerebellum under high magnification (400×). **E:** Luciferase immunostaining of the same cerebellum in D under high magnification. Arrows, location of immunoreactive cell bodies.

activity in the cerebral cortex and the brainstem colocalized with MBP immunoreactivity, not all of the MBP-positive cells were luciferase-positive. In all three regions of the brain, no apparent luciferase immunoreactivity was detectable in the white matter tracts. This lack of luciferase staining may reflect the lack of luciferase expression in a subpopulation of oligodendroglial cells within the white matter. Alternatively, it may be a result of difficulty in the access of antibodies to luciferase antigen in this region. Since relatively high amounts of the lipid membranes were present in the white matter, it may have been more difficult for antibodies to penetrate into the peroxisomes for binding to the luciferase antigen. The fact that all luciferase-positive cells in the cerebral cortex and the brainstem were MBP-positive indicated that the transgene was targeted specifically to oligodendroglial cells in the brain, even though not all of the oligodendrocytes were luciferase-positive.

In examining the distribution of enzymatic activity in the transgenic mice, we found that the relative level of luciferase enzymatic activity in the cerebral cortex did not correspond to that of the endogenous GPDH enzyme activity. In all three lines of the Tg4.3 mice, and in four lines of the Tg2.6 mice, luciferase activity was higher in the cerebral cortex than in other regions of the brain. In some cases the luciferase activity in the cerebral cortex was as high as activity in the fat. This is unlike the endogenous GPDH activity, which is distributed approximately equally in all three regions of the brain, and at least 7-fold lower than in the fat. Since our mixing experiments indicated that the degradation mechanism of each tissue homogenate did not differ considerably from one another, this extraordinarily high level of luciferase activity most likely arises from a higher synthesis rate of the luciferase enzyme in the cortex. Since luciferase immunoreactivity was not detected ectopically in the GPDH-negative neurons and astroglial cells, the luciferase was probably more expressed at a higher level on a per cell basis. It is possible that the 5′ flanking region of GPDH contains a cortex-specific

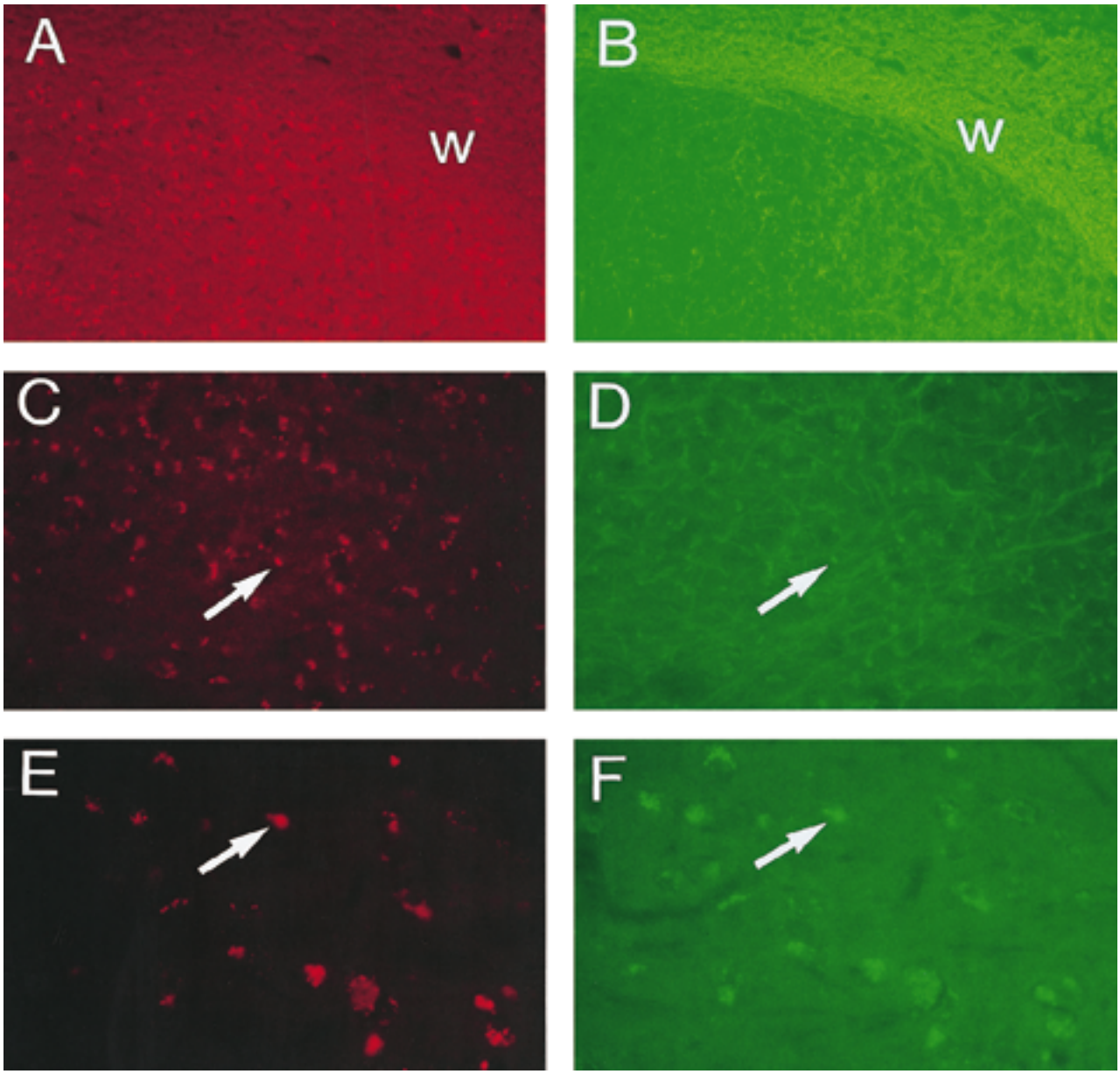

Fig. 6. Double immunolocalization of luciferase and MBP-expressing cells in the cerebrum and the brainstem. **A:** Luciferase immunostaining. **B:** MBP staining of the cerebral cortex. Magnification, 200×. w, white matter tract. **C:** Luciferase immunostaining of the same section in A under high magnification, 400×. **D:** MBP immunostaining of the same section in B under high magnification, 400×. Arrow, location of a representative double-immunoreactive cell. **E:** Luciferase immunostaining of brainstem area under high magnification, 400×. **F:** MBP immunostaining of the same section under high magnification, 400×.

element that is more active in the absence of downstream sequences. Additionally, it is possible that the 5′ flanking sequence contains cell-specific elements from GPDH's upstream gene D15kz1, which is located only 3.8 kb upstream of GPDH exon 1 (Johnston et al., 1989). Although the function of the gene product for D15kz1 is unknown, the message for D15kz1 is known to be expressed in the cerebral cortex. The high level of transgene expression in the cerebral cortex may be related to an enhancer activity associated with D15kz1 expression in the GPDH 5′ flanking sequence. Possibly, an enhancer-related D15kz1 expression in the cerebral cor-

tex increased the transcriptional activity of the GPDH transcription initiation.

In addition to the brain, luciferase activity was also detected in fat, a tissue that normally expresses the highest level of endogenous GPDH gene product. This indicates that a ''fat-specific'' element may be present in the 5′ flanking region of GPDH. Previously, a stretch of sequence homologous to two other fat-specific genes, the adipsin (Hunt et al., 1986) and P2 gene (Phillips et al., 1986), was identified upstream of the mouse GPDH gene. However, this ''fat-specific'' element does not appear to be required for fat-specific expression, as deletion of it did not affect transgene expression in the mice (Birkenmeier et al., 1992). Results of this study showed that the 5′ flanking region alone was sufficient to direct reporter gene expression in fat tissues in vivo, and that this fat-specific enhancer is most likely located within the conserved sequence of the 5′ region.

Previously, Kozak et al. (1990) analyzed the activity of the mouse 5′ flanking region using a transient transfection experiment. Utilizing a chloramphenicol acetyltransferase reporter gene, they found that the 5′ flanking region of the mouse GPDH-encoding gene (Gdc-1) was active in GPDH-negative Hepa and L cell lines. The conclusion they reached was that the GPDH 5′ flanking region was transcriptionally active, even in cells that normally do not express GPDH. Therefore, no tissue-specific elements could be identified in the 5′ flanking region. Similarly, we observed in a previous transfection study that the rat 5′ flanking region was active in GPDH-negative fibroblast cells (Cheng and de Vellis, unpublished results). These two studies predicted that a reporter transgene containing only the GPDH 5′ flanking region would be expressed without any specificity in all cell types. Our transgenic data showed that this was not the case. The reporter transgene was expressed in fat and brain, not in other GPDH-producing or GPDH-negative cells. The discrepancy in specificity of the 5′ flanking region indicates that the transcription activity estimated from transient transfection studies can not be used as a truthful representation of transcriptional activities in vivo. Epigenetic factors, such as hormones, growth factors, and cell-to-cell contact can dramatically change the repertoire of gene expression for cells in tissue culture condition.

Regulation of tissue- and cell-specific gene expression in a multicellular organism is complex. This is especially true for a gene like GPDH whose expression is highly specific in different cell types. Independent regulatory elements are likely to exist in order to regulate the complex transcriptional activity in different tissues. The fact that the 5′ flanking region confers fat- and brain-specific expression indicates that some of these elements function independently, regardless of the intron, and 3′ flanking sequences. Recently, GPDH mRNA and protein were found to be stress-inducible in the rat hippocampus. Experimental stress paradigms such as vibratory stress cause a prominent increase in GPDH mRNA level in oligodendrocytes. The increase is related to increases in endogenous glucocorticoid level (Nichols et al., 1989, 1990, 1996; Masters et al., 1994). Moreover, in the mouse cerebellum, GPDH expression in Bergmann glial cells is also known to be dependent on appropriate contact with neighboring Purkinje cells. Loss of Purkinje cells, or changes in thyroid hormone status have been shown to affect expression of GPDH in Bergmann glial cells (Finch et al., 1990; Fisher, 1984; Sugisaki et al., 1991). This information indicates that GPDH is not only a glial cell-specific marker, but also is a sensitive indicator of changes in glial cell metabolism and adaptation to extracellular environment. In a previous report, we found that the 5′ flanking region of the rat GPDH gene was glucocorticoid-inducible in C6 glioma cells. It is possible that the same glucocorticoid inducibility can be recapitulated under in vivo conditions. The fact that the reporter gene driven by the GPDH 5′ flanking region was expressed specifically in glial cells provides an in vivo model to study the changes in metabolism elicited by extracellular perturbation in oligodendroglial and Bergmann glial cells.

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