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Expression of Green Fluorescent Protein in Oligodendrocytes in a Time- and Level-Controllable Fashion with a Tetracycline-Regulated System

Chang J. Huang, Francesca Spinella, Ramin Nazarian, Matthew M. Lee, Joel M. Dopp, and Jean de Vellis

Departments of Neurobiology and Psychiatry, Brain Research Institute, Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, California, U.S.A.

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Abstract

Developments in transgenic technology have greatly enhanced our ability to understand the functions of various genes in animal models and relevant human diseases. The tetracycline (tet)-regulated transactivation system for inducing gene expression allowed us to control the expression of exogenous genes in a temporal and quantitative way. The ability to manipulate a cell-specific promoter enabled us to express one particular protein in a single type of cell. The combination of a tetracycline system and a tissue-specific promoter has led us to the development of an innovative gene expression system, which is able to express genes in a cell type–specific and time- and level-controllable fashion. An oligodendrocyte-specific myelin basic protein (MBP) gene promoter controls the reversed tet-inducible transactivator. The green fluorescent protein (GFP) gene was placed under the control of the human cytomegalovirus (CMV) basic promoter in tandem with seven tet-responsive elements (TRE), binding sites for the activated transactivator. Upon the addition of doxycycline (DOX, a tetracycline derivative), tet transactivators became activated and bound to one or more TRE, leading to the activation of the CMV promoter and the expression of GFP in oligodendrocytes. We have successfully expressed GFP and luciferase at high levels in oligodendrocytes in a time- and dose-dependent fashion. In the absence of DOX, there was almost no GFP expression in oligodendroglial cultures. Graded levels of GFP expression were observed after induction with DOX (0.5 to 12.5 μg/ml). Our data indicate that this inducible gene expression system is useful for the study of gene function in vivo and for the development of transgenic animal models relevant to human diseases such as multiple sclerosis.

Introduction

Conventional transgenic technology has contributed significantly to the understanding of the functions of genes within the context of living organisms. However, traditional transgenic technology is limited by the fact that transgenes are overexpressed in multiple tissues from conception onward (1). This is particularly problematic in the development of the nervous systems such as the myelin sheath, where changes in the level of one gene induce compensatory changes throughout the system (2). The newest challenge to the transgenic technology is to have the ability

Francesca Spinella's current address is: Institute of Biochemistry and Department of Physiological Science, University of Catania, Viale Doria 6, 95125 Catania, Italy

Address correspondence and reprint requests to: Jean de Vellis, Ph.D., 760 Westwood Plaza, 68-177 NPI, MRRC, UCLA, Los Angeles, CA 90024, U.S.A. Phone: 310-825-0239; Fax: 310-206-5061; E-mail: jdevellis@mednet.ucla.edu

to regulate the expression of a transgene in terms of level, anatomical location, and time (3).

The recent development of a tetracycline (tet)-regulated transactivation system for inducible gene expression makes quantitative, spatial, and temporal induction of transgenes in cells and animals possible (4–6). In the tet system, inducibility of transgene expression is conferred by the transactivator, a reverse-tetracycline repressor/VP16 (rTetR/VP16) fusion protein, which undergoes a conformational change and binds the tet-responsive element (TRE) only in the presence of doxycycline (DOX), an analogue of tetracycline. The TRE, linked to the minimum cytomegalovirus (CMV) promoter, drives transcription of a transgene. The amount of transactivator protein binding and, hence, the level of transgene induction are directly proportional to the concentration of DOX.

Anatomical specificity is achieved by the use of a promoter that is active only in a particular cell type. By placing transcription of the tet-responsive transcriptional factor under the control of a cell-specific promoter, gene expression can be induced in particular types of cells at a specific time. The central nervous system (CNS) is an ideal candidate for the application of this technology. Several cell-specific promoters have been well characterized in the brain. Additionally, the brain undergoes well-delineated developmental stages during which the circumscribed manipulation of a target gene could have functional importance.

To explore the potential for regulated transgene expression in the CNS, we generated a novel gene construct in which transcription of the transactivator gene was placed under the control of the myelin basic protein (MBP) promoter. Myelin basic protein is an integral component of the myelin sheath and is normally expressed only by mature oligodendrocytes. Green fluorescent protein (GFP) and luciferase were used as reporter genes. In transfected MBP^+ human oligodendroglioma (HOG) cells or primary rat oligodendrocytes, the addition of varying concentrations of DOX induced graded expression of luciferase or GFP, respectively. Collectively, the data demonstrate the feasibility of this novel construct for the introduction of inducible transgenes into specific cell populations in the CNS. This technology has significant potential for use in CNS developmental studies and in molecular medicine, especially future gene therapies for humans.

Materials and Methods

Construction of pTetra-MBP-GFP Inducible Expression System

A 6.8-kb pRetro-On plasmid (Clontech Laboratories, Palo Alto, CA) was used as the original vector for all constructs. The pRetro-On was digested with *Not*I and *Bam*HI, and then ligated with a DNA oligonucleotide containing multiple cloning sites (MCS, *Not*I-*Kpn*I-*Apa*I-*Pvu*II-*Nde*I-*Sph*I-*Spe*I-*Bam*HI). The resulting pRetro-MCS was digested with *Pst*I and *Avr*II to remove the retroviral part of the vector sequences. The vector without the *Pst*I–*Avr*II fragment was separated by agarose gel electrophoresis and purified by an Ultrafree-MC 0.45-μm Filter Unit (Millipore, Bedford, MA). The 1.4-kb mouse MBP gene promoter (7) was amplified by a polymerase chain reaction (PCR) and cloned into a pCRII vector (Invitrogen, Carlsbad, CA). The pCRII-MBP was digested with *Nsi*I and *Spe*I to release the 1.4-kb MBP promoter. The purified promoter fragment was cloned into the *Pst*I and *Avr*II sites of pRetro-MCS. The new DNA construct, named pTetra-MBP-MCS, was confirmed by the di-deoxy chain-termination sequencing method. The GFP cDNA (Clontech) was cloned into the *Apa*I/*Spe*I sites of pTetra-MBP-MCS. The final construct, called the pTetra-MBP-GFP (Fig. 1), was verified by restriction endonuclease mapping. As a comparison, the GFP gene was replaced by luciferase to create a pTetra-MBP-LUC construct. The pE-GFP-N2 construct contains a CMV promoter to control GFP expression (Clontech). An Endofree Plasmid Giga preparation kit (Qiagen, Valencia, CA) was used to obtain pure plasmid DNA for transfection. The correct orientation and sequences of all DNA constructs were confirmed by using a di-deoxy chain-termination sequencing method.

Electroporation

Transfection of human oligodendroglioma cells [HOG, (8)], primary rat oligodendrocytes, and astrocytes with pTetra-MBP-GFP, pTetra-MBP-LUC, and pE-GFP-N2 constructs was performed using electroporation. Briefly, oligodendrocytes were harvested by trypsinization. The cells were resuspended at 1×10^6 cells/ml in serum-free Dulbecco's modified Eagle's medium (DMEM). A 700-μl aliquot of cell suspension was placed in a 4-mm gap-electroporation cuvette and mixed with the desired plasmid DNA. The cuvettes were kept at room temperature before electropora-

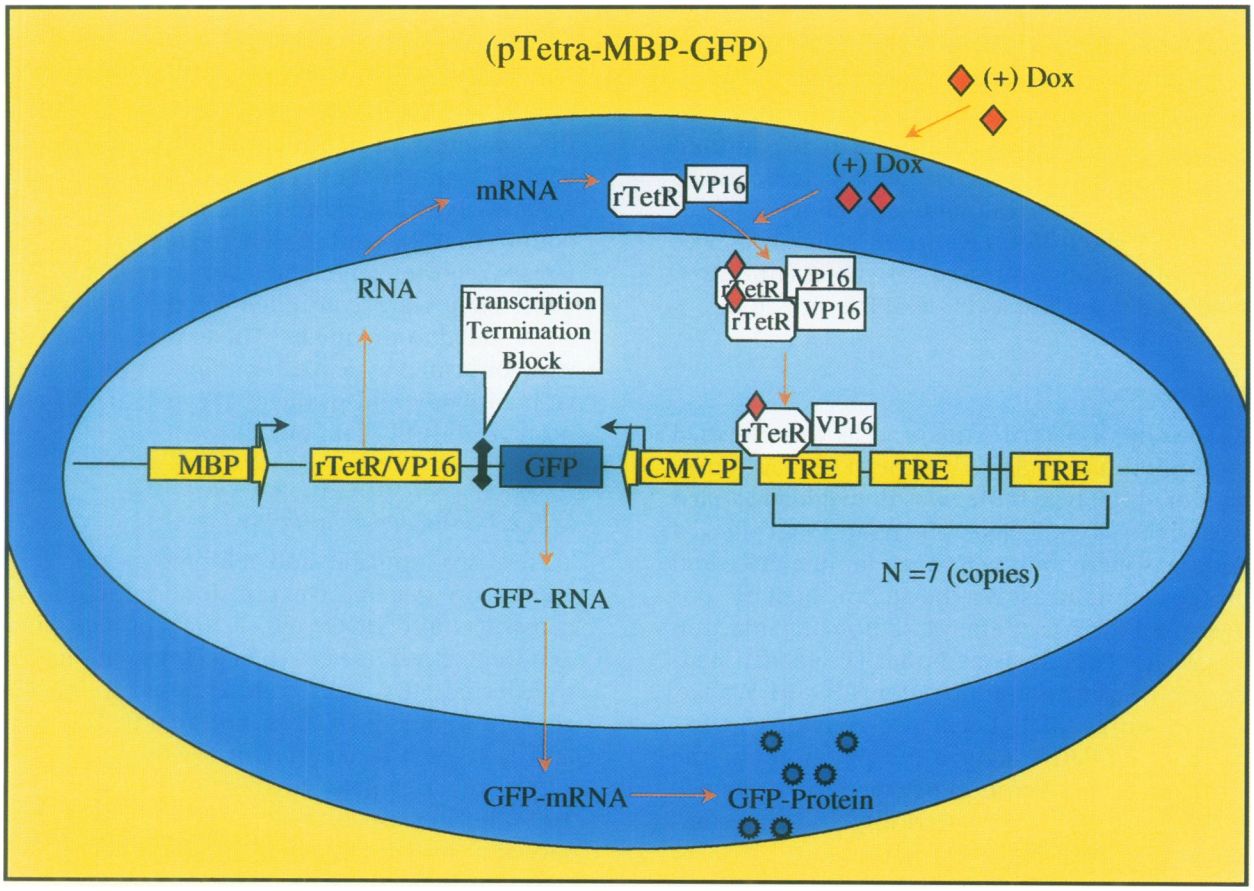

Fig. 1. Schematic diagram depicting the cell-specific, time- and level-controllable expression system. The Tet-inducible transactivator (rTetR/V16) is placed under the control of the myelin basic protein (MBP) gene promoter, restricting its expression to oligodendrocytes. Green fluorescent protein (GFP) is controlled by the human cytomegalovirus (CMV) minimal promoter in tandem with seven tet-responsive elements (TRE), binding sites for the activated transactivator protein. In the presence of doxycycline (DOX), the tet transactivator bound to one or more TRE, leading to the activation of CMV promoter and expression of GFP by oligodendrocytes. There is a transcription termination block between the rtetR/VP16 gene and GFP gene.

tion. Cells were electroporated at 300 V and 800 μF in a BioRad Gene Pulser II electroporator (BioRad Labs, Hercules, CA). Typical duration of discharge ranged from 10 to 30 msec. The percent cell death due to electroporation damage was approximately 10–20% of the total cells being used. Transfected cells were plated in DMEM medium containing 10% fetal calf serum (FCS) at 1×10^5 cells/well in flat-bottomed, poly-L-lysine-coated 24-well plates (Falcon, Lincoln Park, NJ). Cells were allowed to adhere for 4 hr, after which the spent medium was replaced by DNB (9) medium with 4% FCS and 0–12.5 μg/ml of DOX. Cells were cultured for 48 hr, in which GFP fluorescence microphotographs were taken. HOG cells were prepared and electroporated using a similar protocol with HOG medium (8). The same procedure was repeated with rat primary astrocytes using 5% FCS-DMEM. For transfection studies, a CMV-controlled GFP expression vector (the pE-GFP-N2 construct, Clontech) was used as a positive control.

Detection of GFP Fluorescence

A Zeiss fluorescence microscope (Zeiss Axiovert 135 M, Mercury Lamp, Carl Zeiss HBO 100W/Z) with a charge-coupled device was used to observe, record, and analyze GFP expression. A spot cooled color digital camera and software (Diagnostics Instruments, Irvine, CA) were used to acquire the image for data analysis. Purified GFP from Clontech was used to calibrate the settings of the microscope with a long-pass FITC

filter (Zeiss set-10, 490-525). Care was taken to separate the GFP signal from autofluorescence, which can be identified by using a rhodamine filter. The use of a cooled CCD camera permitted quantitation of GFP intensity (10) in cultured cells. The images of GFP^+ cells were captured with the Spot camera mentioned above. The intensity of green fluorescence in single cells was measured and expressed as pixel numbers, which reflected the level of GFP expression.

Luciferase Assay

At the end of DOX treatment, cells were washed with phosphate-buffered saline (PBS). A 100-μl aliquot of cell lysis buffer was then added to each well of the 24-well plate. The lysate was collected and the supernatant was saved for luciferase and protein content assays. Luciferase activity was measured for 10 sec after an automatic injection of 100 μl of firefly assay buffer (Promega, Madison, WI), using a luminometer (LB9501 Wallac, Gaithersburg, MD). Luciferase activity was expressed as relative light units (RLU) above the instrument's background per mg protein, which was determined colorimetrically using a commercial assay kit (Bio-Rad).

Doxycycline Treatment

Doxycycline hydrochloride (Sigma Chemical Co., St. Louis, MO) was prepared as a 10 μg/μl stock in 95% ethanol and was then serially diluted with culture medium as working solution. Various concentrations of working solution were added into the culture medium as indicated in the figure legends.

Results

A series of novel DNA constructs (Fig. 1) were developed by modification of the commercially available retroviral-based vector (pRetro-On). In order to convert this retroviral system to a plasmid DNA vector, we removed the LTR, DNA sequences for packaging signal, SV40 promoter, and puromycin selection marker gene. We replaced them with a cell-specific gene promoter, 1.4 kb myelin basic gene promoter [MBP; (11)] and a reporter gene (luciferase or GFP). This new vector system acquires three features: cell specificity, and time- and level-controllable expression. Cell-type specificity was achieved by using a MBP promoter, which restricted the expression of rTetR/VP16 to oligodendroglial cells only. The inducibility of reporter gene or transgene expression was achieved by addition of the media containing doxycycline, a tetracycline derivative. In the presence of DOX, the ligand-rTetR/VP16 complex underwent conformational changes and was translocated into the nucleus, where it bound to TRE and transactivated the downstream reporter gene. The levels of reporter gene expression were controlled by the amount of DOX added to the media. The expression of GFP was confirmed by a Western blot, using an anti-GFP antibody and purified GFP (Clontech) as a positive control (data not shown).

Cell Specificity and Inducibility

To test this inducible and cell-type specific gene expression system, we transfected these DNA constructs into HOG cells, a transformed oligodendrocyte cell line (8), by electroporation. The transfected HOG cells were exposed to various concentrations of DOX as indicated in Figures 2 and 3. In the first experiment, we used luciferase as the reporter gene to transfect HOG cells. A 100-fold increase in luciferase activity was observed at concentrations ranging from 0 to 100 ng/ml of DOX.

The expression level of the reporter gene, as reflected by the amounts of luciferase activity, was highly correlated ($p < 0.01$) to the levels of DOX added to the culture medium (Fig. 2). Since the product of the luciferase reporter gene and the assay did not identify the source of luciferase-secreting cells, we used GFP as a reporter gene to directly visualize the cells that express GFP and determine its intensity as a function of promoter activity. As shown in Figure 3, the intensity of green fluorescence was highly correlated with the concentration of DOX added into the culture medium. These GFP data are in agreement with the luciferase data that show that the inducible gene expression system is working well in vitro.

After we optimized the conditions of transfection, rat primary oligodendroglial cultures were transfected with pTetra-MBP-GFP DNA construct (Fig. 1). A small number of GFP^+ cells were observed in the absence of DOX (Fig. 4). With increasing concentrations of DOX, the GFP^+ cells became more abundant and reached about 38% GFP expression of the total population (Fig. 3). Increasing levels of GFP expression were observed in the presence of DOX from 0.01 to 12.5 μg/ml. The GFP^+ cells first became visible

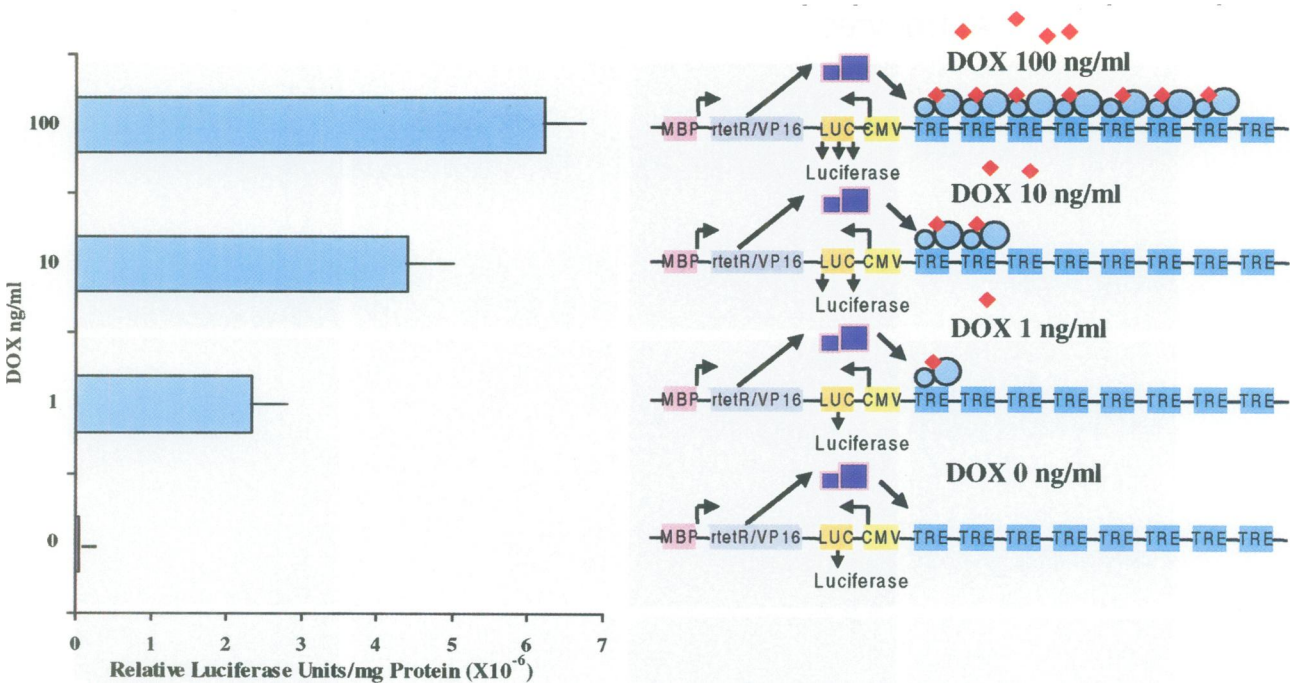

Fig. 2. DOX induces MBP-controlled luciferase expression in a dose-dependent manner. Human oligodendroglioma (HOG) cells were transfected via electroporation with a construct similar to that in Figure 1, using the MBP promoter and luciferase as a reporter gene. Forty-eight hours later, DOX was added at the indicated concentrations and incubated for 24 hr, after which luciferase activity and protein content were analyzed (left panel). Means ± SD of three replicates are shown. Right panel: Schematic illustration of the postulated mechanism of graded luciferase expression. In the presence of more DOX, there are more tet transactivators bound to TREs, leading to a stronger activation of CMV promoter and a greater expression of luciferase by oligodendrocytes.

10 hr after addition of DOX (10.0 μg/ml). These cells remained positive for as long as 1 week, which reflected the long half-life and stability of GFP (12). To demonstrate that GFP was specifically expressed, we transfected pTetra-MBP-GFP DNA construct into mixed glial cultures (containing astrocyte, oligodendrocytes, and microglia) and pure astrocytes. We observed that only oligodendrocytes were GFP^+ by morphological criteria (Fig. 4M). We investigated whether the preferential expression of GFP in oligodendrocytes but not in astrocytes or microglia was due to the promoter specificity and not to the varying transfectability of different cell types. We used CMV promoter–driven GFP and MBP-driven GFP DNA constructs to transfect astrocytes. Astrocytes expressed GFP only under the CMV promoter, but not under the MBP promoter–driven GFP construct (Figs. 4 and 5).

Kinetics of MBP Promoter Activation in the Presence of DOX

The time course of DOX action on MBP promoter activation was analyzed in HOG cell cultures-

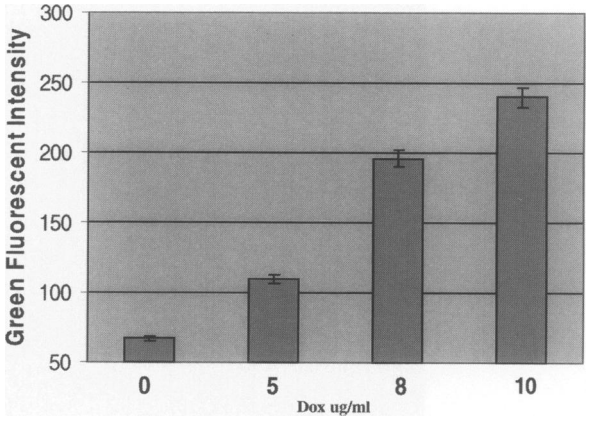

Fig. 3. DOX induces MBP-controlled green fluorescent protein (GFP) expression in a dose-dependent manner. Primary rat oligodendrocytes were transfected via electroporation with a construct similar to that in Figure 1, using the MBP promoter and GFP as a reporter gene. Forty-eight hours later, various concentrations of DOX as indicated in the legend were added for 24 hr, after which the cells were observed and photographed. The green fluorescence intensity from five cells in each field and five fields from each well was measured by a fluorescent microscope with a Spot camera (see text). The experiment was repeated two times.

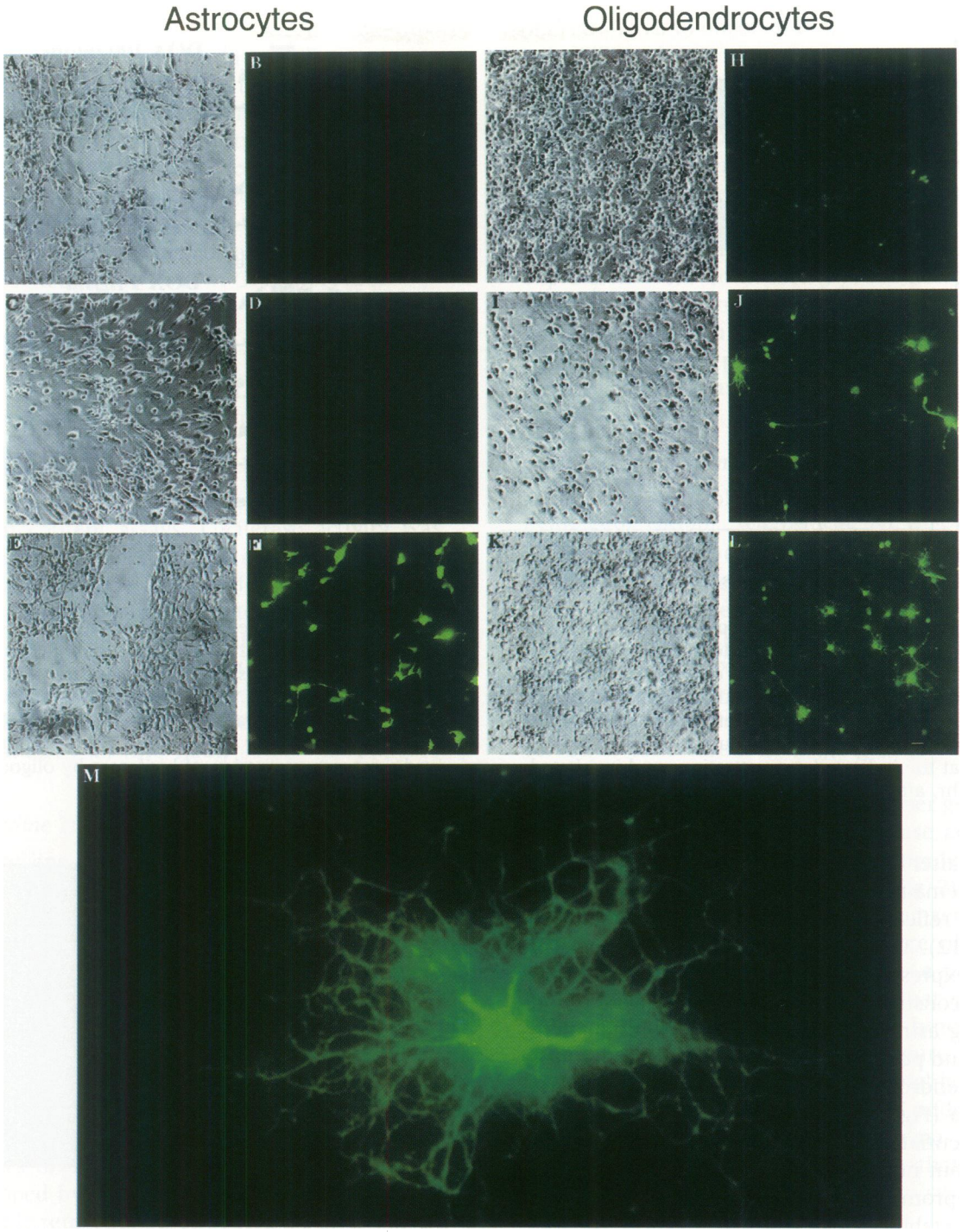

Fig. 4. GFP expression in a dose-dependent and cell-type specific manner. GFP expression in transfected primary glial cells is both inducible and cell-specific. Astrocytes (A–F) and oligodendrocytes (G–L) were transfected via electroporation with pTetra-MBP-GFP (A–D and G–L) or pEGFP-N2 (E–F), incubated for 24 hr with various concentrations of DOX, and photographed by phase (A, C, E, G, I, K) or fluorescent (B, D, E, H, J, L) microscopy. In the absence of DOX, astrocytes expressed little or no GFP (B), whereas oligodendrocytes expressed low basal levels (H). The presence of 5 μg/ml DOX significantly increased the percentage of GFP^+ oligodendrocytes (J) but not GFP^+ astrocytes (D). The percentage of positive cells and the intensity of GFP expression by oligodendrocytes (L) were further increased by 10 μg/ml DOX. The relatively high percentage and intensity of GFP expression by astrocytes transfected with the CMV-GFP construct (F) demonstrated that these cells are readily transfectable. Bar = 20 μm. (M) Enlargement of an oligodendrocyte depicted in L expressing GFP.

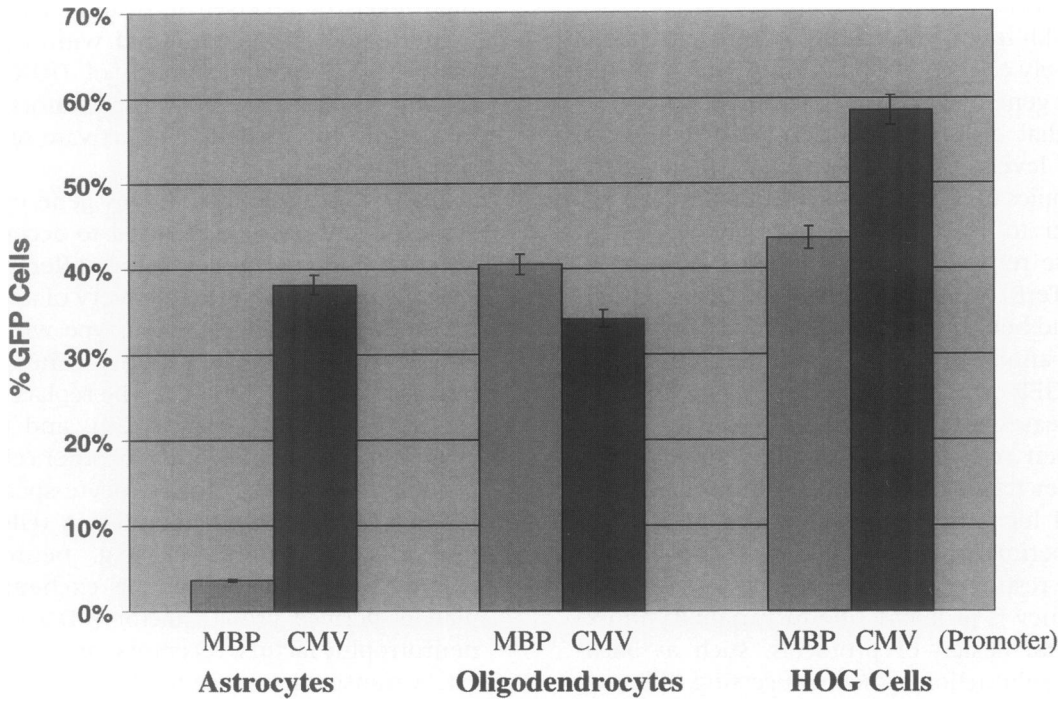

Fig. 5. GFP expression in cell-type specific manner. Bar graph showing expression of GFP in astrocytes, oligodendrocytes, and HOG cells in relation to CMV promoter-driven GFP and MBP-driven GFP DNA constructs. (See Figure 4 for details.)

grown in the absence or presence of DOX. After transfection with the tetra-MBP-GFP construct, DOX was added to the culture medium at the final concentration of 10 μg/ml. The control culture received only vehicle. At various times, GFP^+ cells were counted under a fluorescent microscope and expressed as a percentage of total attached cells. As shown in Figure 6, the maximal expression of GFP occurred at 66 hr after transfection of HOG cells.

Compared to the MBP promoter, the CMV promoter was stronger in the activation of GFP expression in HOG cells. Furthermore, CMV-driven construct expressed GFP earlier than the MBP-driven construct, since the latter construct needs two transcriptions and two translations to express GFP. In the interval of 66 to 90 hr, GFP expression in CMV-GFP and MBP-GFP constructs dropped by 10% and 5%, respectively. The GFP fluorescence from pTetra-MBP-GFP disappeared after 1 week, but GFP^+ cells were still present in relatively high number in the pCMV-GFP transfected cells even after 3 weeks (data not shown).

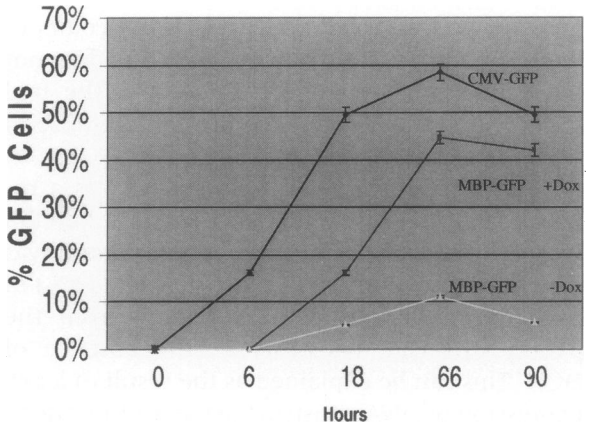

Fig. 6. Kinetics of MBP promoter activation and GFP expression in oligodendroglioma cells. HOG cells were transfected with the inducible expression construct pTetra-MBP-GFP and positive control CMV-GFP, a noninducible construct separately. The transfected cells were exposed to 10 μg/ml of DOX and observed at several time points after transfection or relative to the time of DOX induction.

Discussion

We have successfully expressed GFP and luciferase at high levels in oligodendrocytes cells in a time- and DOX-dose-dependent fashion. Reporter expression was restricted to oligodendrocytes (Fig. 4) by using the 1.4-kb fragment of MBP promoter upstream to the tetracycline-re-

sponsive transactivator gene [rTetR/VP16; (13)]. The 1.4-kb fragment of MBP promoter has been extensively characterized and shown to control a reporter gene or a transgene expression in oligodendroglial cells (7,11,14,15). Inducibility and variable levels of expression were achieved by seven copies of the TRE in tandem with the CMV promoter to drive transcription of the GFP or luciferase reporter genes. Upon the addition of DOX, rTetR/VP16 transactivators become activated and bound to one or more TRE, leading to the activation of the CMV promoter and expression of GFP or luciferase in oligodendrocytes.

Increases in luciferase activity over 1000-fold have been reported with stable transfer of reversed tet transactivator into cell lines (4). The levels of luciferase activity induced by DOX in our experiment were much lower compared with the results reported by others (4–6,16). The discrepancy is probably due to variability in experimental design or protocols, such as background subtraction when processing the raw data. The second possible reason for deviation may be due to the different times of measurement to which the luminometer was set. The value of relative luciferase activity increases with duration of measurement set on the luminometer. In contrast, GFP acts to shift the colors of bioluminescence from blue to green and does not have an amplifying effect. Therefore, the fold induction is smaller and requires a high dose of the inducer, DOX (Fig. 3).

Figure 5 illustrates the use of GFP as a reporter gene for the study of real time kinetics with an inducible expression system. There was an unexpected and relatively high level (11%) of background due to GFP expression from the pTetra-MBP-GFP construct in the absence of DOX. This can be explained as the result of leaky expression of DNA construct per se and/or due to the nonintegrated effect in a transient transfection system. The stable transfer of tet-regulated system into a cell line or integrated into a locus may reduce the problem (17–19). The leaky expression of GFP in the study may be related to the fact that we put the system of cell-specific expression of rTetR/VP16 and the system of tet-responsive promoter with the reporter gene into a single vector system. Most researchers use two separated systems, which have advantages such as flexability to link a gene of interest and tight controlled expression. In mature oligodendrocytes, a 1.4-kb MBP gene promoter is relatively strong in terms of transactivating downstream gene expression (7,11,17). Therefore, relatively high levels of reversed tet-transactivators were accumulated and sequestered within the cytoplasm. Even in the absence of DOX inducer, there is a chance for tet-transactivators to translocate into the nucleus and activate tet-responsive promoter.

Our data indicate that this gene expression system could possibly be used to accommodate different targeted genes using different tissue-specific promoters for the delivery of a therapeutic product to a selective cell type with a time- and level-controllable capacity. The inducible transactivator (tet/VP16) can be replaced by others, such as GAL4 system (20), and the MBP promoter can be substituted by other cell-specific promoters, such as an astrocyte-specific promoter glial fibrillary acidic protein (GFAP) or a neuron-specific promoter [e.g. neurofilament (21)]. The GFP gene can be exchanged with platelet-derived growth factor (PDGF) or other neurotrophic factors, receptors, or genes of interest. A transgenic mouse model using this inducible expression construct is currently being characterized using thioredoxin peroxidase (22) as the reporter gene with an SV40 viral promoter to control the expression of rTetR/VP16.

A system that facilitates expression of a transgene in a spatial, temporal, and quantitative fashion has enormous applications in developmental biology and human gene therapy. From the human genome project, DNA sequences of an estimated 70,000 to 100,000 genes will be made available in the next few years. Our gene expression system could be an ideal tool to study the function of a selective gene when it is expressed in a cell-specific, time- and level-controllable manner. The features of this gene expression system are especially useful for studying the developmental role of a particular gene. For example, if the gene product is toxic or lethal when expressing during early embryonic development, then no live animal would be obtained (23). The inducible expression of a transgene model is very useful in such a situation to avoid potential compensatory developments, defects, or even embryonic mortality. The animals would be normal until the inducer is administered to activate expression of the toxic or lethal gene. Human gene therapy often requires that a specific gene product be expressed in a specific tissue at a time and level determined as optimal by clinical studies. Our gene expression system is ideal for clinical testing in a therapeutical gene approach for specific monogenetic disorders. However, several barriers need to be overcome, such as long-term

gene delivery and stable expression, a major goal of current research in this field.

Acknowledgments

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