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Nonlethal Detection of Transgene Expression in the CNS of Founder Mice

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ABSTRACT

Determining whether transgenes targeted to the central nervous system (CNS) are expressed has previously required sacrificing the animal; thus, if a line was to be maintained, it was necessary to generate offspring from the original founder mouse before assay. We describe a method for assaying for the expression of a variety of CNS-targeted transgenes that allows for the survival of the founder mice. Many CNS genes are expressed in the retina, which can be obtained from a mouse by simple survival surgery. We demonstrate that reverse transcription PCR of RNA isolated from a single eye can detect the mRNAs for glial fibrillary acidic protein (GFAP), vimentin, neurofilament light chain (NF-68) and Thy-1, all of whose promoters have been used to direct transgene expression in the CNS. We also show that this method readily detects expression of an astrocyte-specific GFAP-driven lacZ transgene and a neuron-specific L7-driven lacZ transgene.

INTRODUCTION

Transgenic mice have become a standard tool for the study of gene function and the development of disease models. However, production and maintenance of these mice remains a time-consuming and resource-intensive chore. Once potential founder mice are generated, the first step is to identify those that have successfully incorporated the transgene. This is usually simple to accomplish; for example, by performing polymerase chain reaction (PCR) or Southern blotting on DNA obtained from tail biopsies. However, the next step, determining which of these mice actually express the transgene, is usually more difficult. In many cases, since obtaining the relevant tissues for testing transgene expression requires sacrificing the mouse, it is necessary to generate and test offspring of the founder. This is especially a problem for

transgenes that express in the nervous system. Although Messing et al. (11) exploited tail biopsies for evaluating transgene expression in Schwann cells of the peripheral nervous system, no comparable method exists for the central nervous system (CNS).

We present here a method by which founder mice themselves can be tested in a nonlethal manner for expression of various transgenes targeted to the CNS. By permitting identification of founder mice in which the transgenes are active, considerable time and resources are saved by eliminating the breeding of mice that prove not to express the transgene. The method involves reverse transcription PCR (RT-PCR) of RNA isolated from eye. Removal of an eye is a relatively simple procedure for laboratory mice and, since they primarily depend on other senses, does not produce serious adverse effects. In fact, mice of the commonly used FVB/N strain carry the *retinal degeneration* allele, which renders them blind by early adulthood (18). The eye is useful for detecting CNS gene expression, as it includes the retina, an anatomical extension of the CNS that contains both neuronal and glial cell populations. We show here that RNA isolated from a single mouse eye is sufficient to detect gene products from promoters that have been used to target transgene expression to both astrocytes and neurons.

MATERIALS AND METHODS

Animal Procedures

Animal procedures were approved by the Animal Use and Care Committees of the National Institute of Neurological Disorders and Stroke (NINDS) and the University of Wisconsin, Madison. Mice used carried a *lacZ* coding region under control of either the astrocyte-specific glial fibrillary acidic protein (*GFAP*) promoter [lines Tg(*GFAP*, *lacZ*)Mes1 and Tg(X;*GFAP*,*lacZ*)Mes3 (2)] or the neuron-specific L7 promoter (13). In preliminary experiments, RNA was isolated from retina obtained from mice sacrificed by cervical dislocation. The eye was collected immediately and dissected at room temperature in Dulbecco's phosphate-buffered saline (Life

Technologies, Gaithersburg, MD, USA) to remove the cornea, lens and vitreous. The retina was then removed from the sclera by cutting at the optic disc, frozen on dry ice and kept at -70°C until RNA was isolated. In subsequent experiments, RNA was isolated from whole eyes. For detection of L7-*lacZ* transgene expression, frozen eyes were supplied by Dr. Maureen McCall (University of Kentucky, Louisville). For all other results presented, eyes were obtained by survival surgery. Mice were anesthetized with 0.5 g/kg, IP tribromoethanol (Sigma-Aldrich Chemical, Milwaukee, WI, USA) (15), the area around the right eye was cleansed with 70% ethanol, and the eye was exposed by putting slight pressure on both eyelids. The eye was then removed by cutting the optic nerve and extraocular muscles. If bleeding occurred, the orbit was packed with Gelfoam™ (Pharmacia & Upjohn, Kalamazoo, MI, USA) to promote clotting. To achieve temporary protection for the eye socket during the post-operative period and until reproductive performance could be tested, a partial tarsorrhaphy was performed using wound clips, and the animals were placed on a warming plate for recovery. Wound clips were removed within 10 days. All animals processed in this manner survived without incident; however, we noted that debridement of the eyelid margins would be necessary for permanent closure of skin over the socket, as might be desired for valuable founder mice that are to be kept for an extended period of time. Collected eyes were promptly frozen on dry ice and kept at -70°C until used for RNA isolation.

RT-PCR

Total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method using RNA STAT-60™ (Tel-Test "B" Inc. Friendswood, TX, USA). Following isolation, the RNA was treated with RNase-free DNaseI (Boehringer Mannheim, Indianapolis, IN, USA) to cleave any contaminating DNA. The digestion was at 37°C for 20 min and used 1 U of DNaseI for up to 10 µg of RNA. The DNaseI was then inactivated by incubation at 65°C for 10 min in the presence

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Table 1. Description of Deoxyoligonucleotide Primers Used for PCR

Gene	Direction	Sequence	Product Length from		Position ^a	Reference ^b
			cDNA	Genomic DNA		
<i>GFAP</i>	forward	TTCCTGTACAGACTTTTCTCC			5416-5435	
	reverse	CCCTTCAGGACTGCCTTAGT	641 bp	3669 bp	9084-9065	X02801
<i>LacZ-mP1</i>	forward	CATGTATACCCCGTACGTCT				
	reverse	GACTTGCTATTCTGTGCATC	508 bp	600 bp		Reference 2
<i>vimentin</i>	forward	ACGCCATCAACACTGAGTTC			209-228	
	reverse	CTGTCTGAATGACTGCAGGG	356 bp	902 bp	1110-1091	Y07738
<i>Thy-1</i>	forward	ATGAACCCAGCCATCAGCGT			555-574	
	reverse	ATTGGAGCTCATGGGATTCTG	356 bp	758 bp	1498-1479	M11160
<i>NF68</i>	forward	TGGAGAATGAGCTGAGAAGC			2570-2589	
	reverse	TTCGTAGCCTCAATGGTCTC	327 bp	711 bp	3280-3261	M13016
<i>L7-lacZ</i>	forward	CGACCAGCGTGTAACAGTTA				
	reverse	ATCGCACTCCAGCCAGCTTT	325 bp	1269 bp		Reference 13

^aPositions are indicated if numbered sequences have been published. For the *GFAP-lacZ* transgene, the forward primer is in the *lacZ* coding region, and the reverse primer is in the *mP-1* segment, downstream of the *mP-1* intron. For the *L7-lacZ* transgene, the forward primer is in exon 3 of the L7 gene, and the reverse primer is in the *lacZ* coding region, which is embedded in exon 4 of the L7 gene.

^bLiterature reference or the GenBank® Accession number.

Figure 1. RT-PCR of RNA from eye. (A) RT-PCR for *GFAP* mRNA. Products of the expected size of 641 bp were produced from RNA isolated from a whole eye of a Tg(*GFAP-lacZ*)Mes1 *GFAP-lacZ* transgenic mouse (TG +) or a non-transgenic litter-mate (TG -) (lanes 1 and 3). Lanes 2 and 4 are negative controls from which the reverse transcriptase (RT) was omitted. The migration positions of size standards are indicated on the left-hand side. (B) RT-PCR for *lacZ-mP1* mRNA. RNA from a whole eye of a Tg(*GFAP-lacZ*)Mes1 *GFAP-lacZ* transgenic mouse yielded a product of about 506 bp, the size expected from the spliced mRNA (lane 1). No signal was produced from RNA isolated from a wild-type litter-mate (lane 3) or in the absence of RT (lanes 2 and 4). The injection fragment used for making the transgenic mice, which contains the intron, was used as a positive-control template (P) (lane 5). The migration positions of DNA size standards are shown on the left. (C) RT-PCR for vimentin (V; 356 bp), *Thy-1* (T; 356 bp) and *NF-68* (N; 327 bp) mRNAs. Products of the expected sizes (Table 1) for properly spliced vimentin (V; 356 bp), *Thy-1* (T; 356 bp) and *NF-68* (N; 327 bp) mRNAs were produced from RNA from eye. No signals were seen in the absence of RT. In each case, the products were confirmed to be authentic by restriction digestion (data not shown). (D) RT-PCR for *L7-lacZ* mRNA. RNA from a whole eye of an *L7-lacZ* transgenic mouse yielded a product of about 325 bp, the size expected from the spliced mRNA (lane 1). No signal was produced in the absence of RT (lane 2). In addition to the results shown here, RNA from four other Tg(*GFAP-lacZ*)Mes1 mice were assayed for *GFAP* and *lacZ-mP1* mRNAs, and RNA from one of these also assayed for vimentin, *Thy-1* and *NF-68* mRNAs, all with comparable results. Comparable results also were obtained using RNA isolated from whole eyes or retinas of several Tg(X;*GFAP-lacZ*)Mes3 mice, which express a different *GFAP-lacZ* transgene.

of 25 mM EDTA. Approximately 3 µg of total RNA were obtained from each retina, and 15 µg of total RNA were obtained from each whole eye.

RT was performed with SUPER-SCRIPT™ II (Life Technologies, Gaithersburg, MD, USA) and oligo(dT) primer according to the manufacturer's protocol, except that the reaction volume was scaled down by one-half to 6 µL, and 2.5 µg of total RNA were used as template. The reverse transcriptase was denatured by heating at 70°C for 15 min, and then 1.0 µL was used as template in a 20-µL PCR run in a GeneAmp® PCR System Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). Reactions were run through 35 cycles of 1 min at 94°C, 1 min 40 s at 60°C and 1 min 20 s at 72°C; followed by a final extension step of 7 min at 72°C. Table 1 shows primer sequences and the expected product lengths.

RESULTS AND DISCUSSION

Survival Surgery

Removal of an intact eye from a

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mouse by survival surgery is a technically simple procedure that is well tolerated by the animal. However, to verify its applicability to assays for transgene expression, individual eyes were obtained by survival surgery from two male and three female 23-day-old mice as described in Materials and Methods. Within 30 min following the surgery, all five of these mice became fully ambulatory and began eating and drinking normally. During their subsequent maintenance, none showed signs of infection or other complications. Reproductive behavior was also normal; four of the five mice were successfully bred by seven weeks of age, which was within four weeks of surgery. This fertility rate is typical for the colony.

Detection of *GFAP* mRNA

As a primary focus of our laboratory is the study of transgenes driven by the astrocyte-specific *GFAP* promoter (3), we first conducted a feasibility experiment by determining if *GFAP* mRNA could be detected in retina RNA obtained from sacrificed mice. To conserve RNA, the RT reaction was scaled down to 6 μ L (half of the volume recommended by the manufacturer) and was performed using 2.5 μ g of total RNA and oligo(dT) as primer. Then PCR was performed using a *GFAP* primer pair separated by introns, so that the product derived from cDNA could be readily distinguished from that derived from any remaining genomic DNA (see Table 1 for the sequences of all primers used and expected product sizes). The RT-PCR from retina RNA produced a band of the expected size (data not shown). Since it is somewhat tedious to dissect the retina from the eye, we next determined if *GFAP* mRNA could be detected in RNA isolated from whole eye. As shown in Figure 1A, this simplified procedure also yielded the expected RT-PCR product. RNA isolated from whole eyes was consequently used for subsequent experiments.

RT-PCR of *lacZ* mRNA from a *GFAP-lacZ* Transgenic Mouse

Since we were able to detect the endogenous transcript of the *GFAP* promoter, we next determined if mRNA could be detected from a *GFAP*-driven transgene. For this experiment, we used line Tg(*GFAP*,*lacZ*)Mes1 (2), which carries a *lacZ* coding region under control of a 2.2-kb promoter from the human *GFAP* gene. This is a particularly demanding test, as the transgenic mRNA is apparently present at very low level since we have been unable to detect it in this line using either northern blotting or RNA probe protection (M. Brenner, unpublished observations). (The low level of the *lacZ* mRNA is probably due to its instability, as we have detected transcripts from other *GFAP*-driven transgenes by these methods; e.g., see References 6 and 7). Since the *lacZ* gene had been joined at its 3' end to a segment of the mouse *protamine-1* gene (*mP-1*) to supply an

intron and polyadenylation signal, it was again possible to use PCR primers that flanked an intron. This was done so that the expected product from properly spliced mRNA (506 bp) could be distinguished from that from genomic DNA (600 bp). RNA from the eye of a wild-type, litter-mate mouse was used as a negative control. As shown in Figure 1B, RT-PCR yielded a clear single band from the eye of the transgenic mouse of the size expected (506 bp) for the spliced mRNA.

To determine if RT-PCR of whole-eye RNA can be used to detect expression of transgenes driven by promoters other than *GFAP*, we surveyed the endogenous mRNAs of the *Thy-1*, vimentin and 68-kDa neurofilament subunit (*NF-68*) genes, as the promoter of each of these genes has been used to drive transgene expression in different cell types in the brain (5,8,10). The NF-68 and Thy-1 proteins are present in many types of neurons (4,9,12). In the eye, NF-68 is found in retinal ganglion cells and horizontal cells (4,17), and Thy-1 is expressed in retinal ganglion cells and amacrine cells (1,14). Vimentin occurs in several cell types, and in the retina, it is found in astrocytes, horizontal cells and Müller cells (4,16,17). To detect each of the corresponding mRNAs, intron-flanking PCR primers were again used (Table 1). As shown in Figure 1C, expression of each of these genes was readily detected in RNA isolated from eye. Finally, we tested whether expression of a transgene driven by a neuronal promoter could be detected. For this, we chose the L7-*lacZ* transgene, which expresses specifically in cerebellar Purkinje cells and retinal bipolar neurons (13). Again using oligonucleotide primers flanking an intron (Table 1), the expected cDNA product was observed (Figure 1D). Since the fragment amplified is over 3 kb from the 3' end of the transgenic mRNA, this also is a severe test of the procedure.

In summary, we have presented a method that makes it possible to detect the expression of transgenes in astrocytes and neurons without having to sacrifice the mouse. The method is simple, rapid and highly sensitive. Its use will obviate the need to breed founders to obtain offspring for this purpose,

providing significant savings in time and resources.

REFERENCES

1. **Barnstable, C.J. and U.C. Dräger.** 1984. Thy-1 antigen: a ganglion cell specific marker in rodent retina. *Neuroscience* 11:847-855.
2. **Brenner, M., W.C. Kisseberth, Y. Su, F. Besnard and A. Messing.** 1994. GFAP promoter directs astrocyte-specific expression in transgenic mice. *J. Neurosci.* 14:1030-1037.
3. **Brenner, M. and A. Messing.** 1996. GFAP transgenic mice. *Methods* 10:351-364.
4. **Chien, C.L. and R.K. Liem.** 1995. The neuronal intermediate filament, alpha-internexin is transiently expressed in amacrine cells in the developing mouse retina. *Exp. Eye Res.* 61:749-756.
5. **Cicart, P., B. Schwartz, A. Vandewalle, M. Bens, C. Delouis, J.-J. Panthier, S. Pournin, C. Babinet and C. Paulin.** 1994. Immortalization of multiple cell types from transgenic mice using a transgene containing the vimentin promoter and a conditional oncogene. *Exp. Cell Res.* 214:35-45.
6. **Delaney, C.L., M. Brenner and A. Messing.** 1996. Conditional ablation of cerebellar astrocytes in postnatal transgenic mice. *J. Neurosci.* 16:6908-6918.
7. **Galbreath, E., S.-J. Kim, K. Park, M. Brenner and A. Messing.** 1995. Over-expression of TGF- β 1 in the central nervous system of transgenic mice results in hydrocephalus. *J. Neuropathol. Exp. Neurol.* 54:339-349.
8. **Leconte, L., O. Semonin, A. Zvara, S. Boisseau, C. Poujeol, J.-J. Julien and M. Simonneau.** 1995. Both upstream and intragenic sequences of the human neurofilament light gene direct expression of lacZ in neurons of transgenic mouse embryos. *J. Mol. Neurosci.* 5:273-295.
9. **Liem, R.K.H.** 1993. Molecular biology of neuronal intermediate filaments. *Curr. Opin. Cell Biol.* 5:12-16.
10. **Lüthi, A., H. van der Putten, F.M. Botteri, I.M. Mansuy, M. Meins, U. Frey, G. Sansig, C. Portet et al.** 1997. Endogenous serine protease inhibitor modulates epileptic activity and hippocampal long-term potentiation. *J. Neurosci.* 17:4688-4699.
11. **Messing, A., R.R. Behringer, J.P. Hamman, R.D. Palmiter, R.L. Brinster and G. Lemke.** 1992. P0 promoter directs expression of reporter and toxin genes to Schwann cells in transgenic mice. *Neuron* 8:507-520.
12. **Morris, R.J.** 1985. Thy-1 in developing nervous tissue. *Dev. Neurosci.* 7:133-160.
13. **Oberdick, J., R.J. Smeyne, J.R. Mann, S. Zackson and J.I. Morgan.** 1990. A promoter that drives transgene expression in cerebellar Purkinje and retinal bipolar neurons. *Science* 248:223-226.
14. **Perry, V.H., R.J. Morris and G. Raisman.** 1984. Is Thy-1 expressed only by ganglion cells and their axons in the retina and optic nerve? *J. Neurocytol.* 13:809-824.
15. **Papaioannou, V.E. and J.G. Fox.** 1993. Efficacy of tribromoethanol anesthesia in mice. *Lab. Anim. Sci.* 43:189-192.
16. **Pixley, S.K., Y. Kobayashi and J. de Vellis.** 1984. A monoclonal antibody against vimentin: characterization. *Brain Res.* 317:185-199.
17. **Shaw, G. and K. Weber.** 1984. The intermediate filament component of the retina: a comparison between different mammalian species. *Eur. J. Cell Biol.* 33:95-104.
18. **Taketo, M., A.C. Schroeder, L.E. Mobraaten, K.B. Gunning, G. Hanten, R.R. Fox, T.H. Roderick et al.** 1991. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA* 88:2065-2069.

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