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Transgenic studies of peripheral and central glia

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ABSTRACT Transgenic manipulation of gene expression in the nervous system has proven immensely useful for the study of glia. This review focuses on studies of Schwann cell and astrocyte biology and pathology. These studies began with promoter mapping for glial-specific genes (P_0 and *GFAP*), and then progressed to oncogene-induced transformation and toxin-induced cell ablation of glia. For GFAP, an intermediate filament of astrocytes, we have investigated the effects of alterations in gene dosage, both in terms of deficiency or excess of this structural protein. Finally, the utility of green fluorescent protein as a marker for live astrocytes is described.

KEY WORDS: *astrocytes, Schwann cells, GFAP, PO*

When transgenic technology was first developed it had immediate appeal in neuroscience. Conventional methods of gene transfer were limited for studies on the nervous system due to the anatomical complexity of the tissue, the shortcomings of cell culture systems, and a dearth of actively dividing cells. Reviews of the impact that transgenics have had on neuroscience have appeared elsewhere (Rossant, 1990), and are beyond the scope of this report. Of the several cell types in the nervous system, however, neurons usually get the most attention, whereas our interests have generally focused on the non-neuronal populations of cells. The goal of this review is to discuss the early work by the Brinster and Palmiter laboratories on tumors of the central nervous system, describe how this led into studies on Schwann cells of the peripheral nervous system, and then relate the course that this work has taken in my own laboratory in recent years.

My initial collaboration with the Brinster and Palmiter laboratories was prompted by the unexpected finding that transgenic mice carrying the SV40 early region developed brain tumors (Fig. 1) (Brinster *et al.*, 1984), and the involvement of an experimental neuropathologist was deemed useful. We quickly determined that the tumors were arising from the choroid plexus epithelium (a suggestion originally made by Bill Hickey). This result was striking on two accounts; mice naturally have a low incidence of spontaneous intracranial tumors, and in those species (such as people) with higher incidences, gliomas are much more commonly found than choroid plexus tumors. Despite the limitations of a tissue such as choroid plexus for many types of experimental studies, Terry van Dyke exploited this and similar models with great success to explore the mechanisms of transformation by T-antigen (Chen and Van Dyke, 1991; Chen *et al.*, 1992).

Given the apparent predilection for choroid plexus exhibited by the SV40 early region (the basis for which has not yet been established), it is of interest whether similar tumors occur in non-human primates (the natural hosts) infected with the parent virus. However, only a demyelinating disease comparable to progressive multifocal leukoencephalopathy (caused by the JC papovavirus in people) has been observed, and then only rarely (Holmberg *et al.*, 1977; Horvath *et al.*, 1992). Whether SV40 can infect humans and cause disease is still the subject of some controversy, given prominence due to the inadvertent administration of the virus as a contaminant of some of the early poliovirus vaccines in the 1950's. No increase in tumors has been detected in the population given the vaccine (Strickler *et al.*, 1998). Recently, SV40 T-antigen sequences were detected in a remarkably high percentage of choroid plexus and ependymal tumors in children (Bergsagel *et al.*, 1992). Others have not yet duplicated this finding, but if correct it will prove a major advance in understanding the etiology of these tumors.

While the choroid plexus tumor phenotype in the SV40 transgenic mice was fascinating, of equal interest to me was the syndrome displayed by a group of mice carrying a combination between the metallothionein regulatory region and the SV40 early region. When the SV40 enhancer region was modified to delete the 72bp repeats, the pattern of gene expression, and resulting pathology,

Abbreviations used in this paper: SV40, simian virus-40; MT, metallothionein; CAT, chloramphemicol acetyltransferase; CNS, central nervous system; GFAP, glial fibrillary acidic protein; TK, thymidine kinase; NMDA, N-methyl-D-aspartic acid; GFP, green fluorescent protein.

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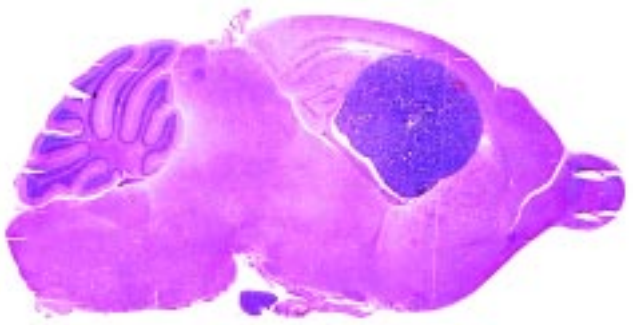

Fig. 1. Parasagittal section through the brain of an MT-SV40 transgenic mouse showing large choroid plexus papilloma arising in the lateral ventricle.

changed dramatically (Messing *et al.*, 1985). Instead of primarily involving choroid plexus, thymus, and kidney, the new major sites were liver, endocrine pancreas, and peripheral nerve. The peripheral nerve lesion resulted from expression of the T-antigens in Schwann cells. When the deleted region also involved the 21bp repeats, expression was eliminated in Schwann cells. Although these mice still developed a peripheral neuropathy resulting in weakness and paralysis, it reflected axonal degeneration rather than demyelination and occurred secondary to hypoglycemia from insulin-producing islet cell tumors (Dyer and Messing, 1989).

Understanding how expression of SV40 was regulated in these modified MT-SV mice so as to target different populations of cells with remarkable specificity was a fascinating question. Another concept originally suggested by Larry Swanson, in collaboration with the Brinster laboratory, was that of "combinatorial specificity", whereby the specificity of expression reflects some interaction between regulatory and coding sequences (Swanson *et al.*, 1985). Although the molecular basis for this idea has never been established, instances keep cropping up that could be explained by such a mechanism. However, pursuing the mechanism of demyelination in mice with such complex patterns of expression and multiple organ involvement proved difficult, and prompted the search for cell-specific promoters that would limit the phenotype appropriately to myelinating glia.

Although Schwann cells are the sole glial cell in the peripheral nervous system, they are not a homogenous population of cells. Those Schwann cells that ensheath large diameter axons produce myelin, whereas those associating with small diameter axons (or the satellite cells surrounding cell bodies in ganglia) remain non-myelinating. These two populations of Schwann cells show dramatic differences in expression of various genes, most conspicuously of those for proteins localized in the myelin sheath (Mirsky and Jessen, 1996). It therefore seemed appropriate to begin looking for a promoter that would limit expression of heterologous genes to myelinating Schwann cells, and the major protein of peripheral myelin is a transmembrane glycoprotein known as P_0. Greg Lemke's laboratory had recently cloned the rat P_0 gene, and identified a relatively small region of 5' flanking DNA that could direct expression of the CAT reporter gene in primary Schwann cells but not in other cell types (Lemke *et al.*, 1988).

We decided to test whether this P_0 fragment could successfully direct expression of a reporter gene in the much stricter test system of transgenic mice (Messing *et al.*, 1992). The human growth hormone gene was selected as a reporter, having previously been used in other cell types such as pancreatic acinar cells (Ornitz *et al.*, 1985). Screening for expression in founder animals seemed desirable, if we could only figure out an innocuous biopsy site for peripheral nerve that did not inordinately compromise neurologic function. While tail sampling one day we realized that the small nerve branches running longitudinally down the length of the tail made these samples an ideal site for evaluating expression in the peripheral nervous system (Figs. 2 and 3). Because these nerve branches are small, however, and difficult to dissect away from surrounding tissues, a morphologic assay such as immunohistochemistry was necessary. The unavoidable presence of non-neural tissues in these samples also offered simultaneous sampling of negative tissues. Again, human growth hormone proved valuable as a reporter since immunohistochemical detection was possible even in plastic-embedded specimens. After identifying founders that expressed growth hormone protein in myelinating but not non-myelinating Schwann cells based on the tail sampling, we established breeding lines from just these animals and later demonstrated specificity of expression at the mRNA level in multiple tissues by northern blots.

We next wanted to apply the still relatively new technique of genetic ablation, originally developed by the Brinster and Palmiter laboratories for the exocrine pancreas (Palmiter *et al.*, 1987) and at the same time used for lens epithelium by Breitman and colleagues (Breitman *et al.*, 1987). Schwann cells are widely distributed throughout the body, and maintain intimate contacts with neurons in all these locations. This anatomic complexity made genetic ablation very appealing as a novel way to perturb Schwann cell-neuron interactions and create an animal model of diffuse hypomyelination. In addition, spontaneous mutants affecting Schwann cell myelination were already available for comparison (Falconer, 1951; Low, 1976). The same wild-type diphtheria toxin

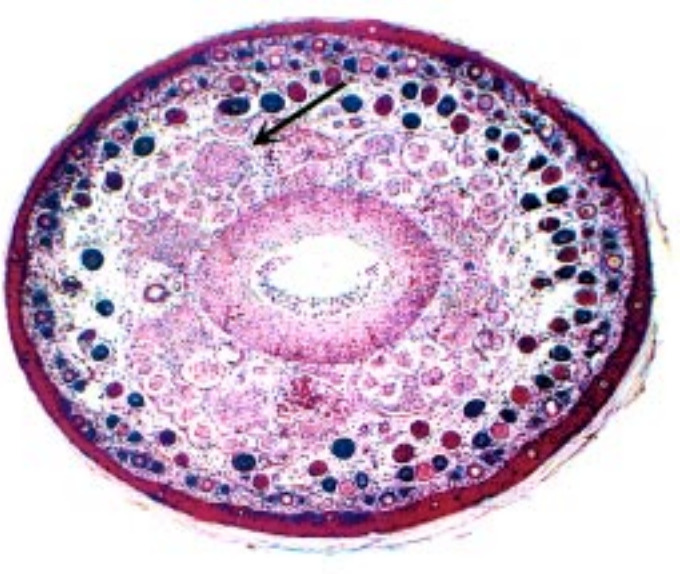

Fig. 2. Transverse section through the distal tail of a weanling mouse, sampled approximately 1 cm from the tip. *One of several nerve bundles is indicated by the arrow. Hematoxylin and eosin stain.*

construct previously used in pancreas was placed under the control of the P_0 promoter, and founder mice were made (Messing *et al.*, 1992). Several died at or soon after birth, and the remaining animals initially looked disturbingly normal. However, evaluation of nerve branches in the tail biopsies revealed subtle but distinct changes in the Schwann cells of one of the surviving founders, who upon breeding produced offspring with a severe neurologic phenotype. Thanks to the microsurgical talents of Richard Behringer in the Brinster laboratory, a stable breeding line was eventually established from this founder by transplanting her ovaries into non-transgenic recipients, and the line was then maintained in this fashion. Perhaps the most interesting result arising from these studies was the finding that the non-myelinating population of Schwann cells began proliferating in response to the cell death taking place in the myelinating population of cells, thus offering a potential reservoir of cells that might be tapped for promoting remyelination.

A third set of studies on myelinating Schwann cells re-visited the questions posed by the MT-SV mice with demyelinating neuropathy described above. In particular, it seemed odd that expression of the SV40 T-antigens, which were considered potent transforming genes for all rodent cells, caused demyelination rather than tumors in Schwann cells. Analogous work at the same time by Judy Small and colleagues using the JC viral early region demonstrated hypomyelination rather than transformation in the myelin-forming oligodendrocytes of the CNS (Small *et al.*, 1986). When transgenic mice were made expressing the SV40 early region under the control of the P_0 promoter, hypomyelination and demyelination were the most prominent features of the neuropathy (Messing *et al.*, 1994). However, the longest lived line (actually expressing the lowest levels of T-antigen) did eventually develop Schwann cell tumors. It is likely that transformation of Schwann cells, as in other cell types, requires one or more secondary events beyond initial expression of the T-antigens, and that the highest expressing lines were simply dying too young for transformation to be completed. Questions that remain unsettled are identifying the precise mechanism of the neuropathy and determining whether Schwann cell proliferation is a consequence or a cause of the myelination defect. Tennekoon *et al.* (Bharucha *et al.*, 1994) have reported direct suppression of myelin-specific genes by T-antigen in cultured cells, which may partly account for the hypomyelination.

After this series of studies focused on the myelinating population of Schwann cells, we then chose to address the fundamental question of how the switch is made from a non-myelinating to a myelinating phenotype. Previous work by Aguayo and others had demonstrated that these two populations are essentially interchangeable, with the interaction with axons being a key determinant of whether myelination takes place (Aguayo *et al.*, 1976). With the long-range goal of modifying gene expression in non-myelinating Schwann cells so as to initiate the full program of myelination, we began a series of studies on the promoter for a gene known to be expressed in the non-myelinating population, glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament that is expressed at highest levels in astrocytes of the CNS, but is also found in several other isolated cell types such as enteric glia and non-myelinating Schwann cells (Jessen and Mirsky, 1984). By this time the bacterial *lacZ* gene had become the standard reporter gene for transgenic studies. Unfortunately for our original goal, the GFAP promoter proved very active for astrocytes, but only gave inefficient

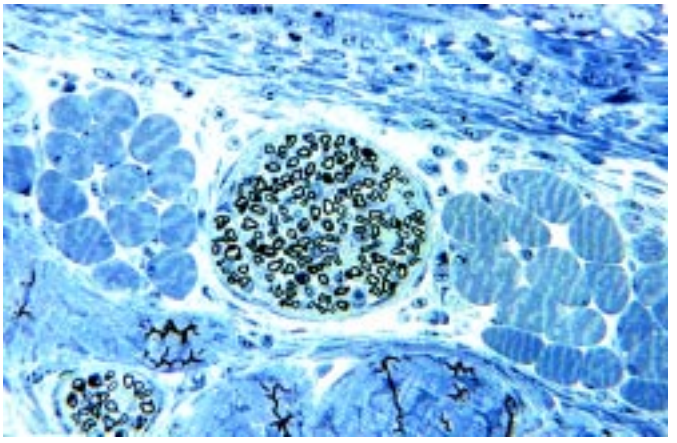

Fig. 3. Plastic-embedded transverse section of tail. *With nerve bundle in the center surrounded by connective tissue, tendons, and muscle.*

expression in non-myelinating Schwann cells (Fig. 4) (Brenner *et al.*, 1994). We therefore began to exploit the *GFAP* promoter for studies where it was useful, i.e., – the astrocyte.

As genetic ablation had proven useful for studies in the PNS, we thought it would be interesting to study the effects of astrocyte depletion in the CNS as well. However, we considered that ablation of astrocytes from the developing CNS using diphtheria toxin would simply create an embryonic lethal. An alternative “conditional” toxin was developed by Evans and colleagues (Borrelli *et al.*, 1989; Heyman *et al.*, 1989), based on the same herpes thymidine kinase that Brinster and Palmiter had used as a reporter in their original studies on gene transfer into the mouse genome (Brinster *et al.*, 1981,1982). Expression of the herpes TK by itself is innocuous, as befits a good reporter gene, but anti-herpetic drugs such as ganciclovir are converted by the viral enzyme into toxic metabolites that interfere with DNA replication and kill proliferating cells. To study the effects of ablating astrocytes at various times during development, we generated GFAP-TK transgenics and then administered ganciclovir as single injections to pups at various times during the first postnatal week (Delaney *et al.*, 1996). The mice were then allowed to survive until postnatal day 19. At that time, the treated transgenics showed obvious ataxia, and histological examination of the cerebellum revealed markedly reduced foliation with decreased numbers of astrocytes and nearly complete elimination of granule cell neurons. A major factor contributing to the death of the granule cell population was excitotoxicity through activation of NMDA receptors. These results are consistent with astrocytes playing a critical role in cerebellar development. However, one of the persisting issues regarding TK-ganciclovir ablation is that of “bystander” killing, whereby non-TK expressing cell acquire the toxic metabolite and die even though they themselves did not express the transgene. Although the mechanism of bystander killing is still the subject of debate, considerable evidence supports a role for transfer through gap junctions (Dilber *et al.*, 1997). Bystander killing is a desirable trait in the setting where it has been most intensively studied, gene transfer for the treatment of solid tumors, but it seriously complicates the analysis of certain phenotypes when applied for selective cell killing in transgenics.

Just as the bacterial *lacZ* gene largely supplanted the first generation of reporter genes (herpes TK, human growth hormone),

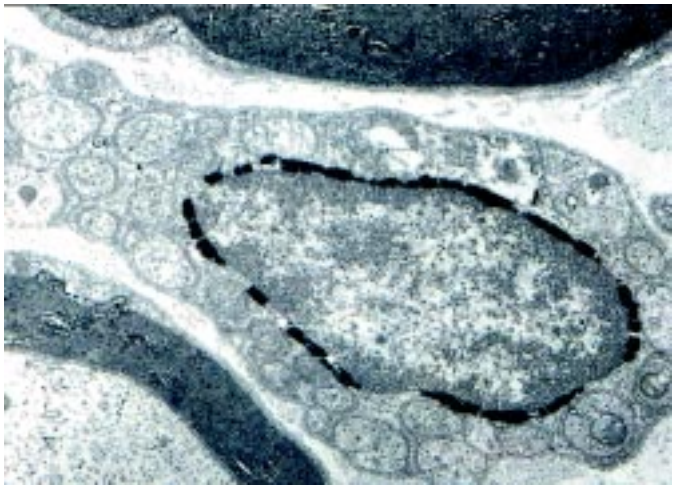

Fig. 4. Electron micrograph of X-gal stained sciatic nerve from mouse carrying a nuclear *lacZ* under the control of the GFAP promoter (Brenner *et al.*, 1994, Tg7.2 line). *The labeled cell in the center is a non-myelinating Schwann cell.*

recently the jellyfish green fluorescent protein (GFP) is proving its worth as a reporter of gene transcription (Cubitt *et al.*, 1995). After the initial description of its use in *C. elegans* (Chalfie *et al.*, 1994), the value of reporters that can be visualized even in living cells became readily apparent. To complement our early studies of astrocyte morphology using *lacZ*, we placed the GFP under the control of the human *GFAP* promoter (Zhuo *et al.*, 1997). Our first experiments used the wild-type GFP, and the fluorescent signal was barely detectable. However, an abundance of GFP mutants were rapidly being generated by many laboratories, and subsequent transgenic mice carrying the hGFP-S65T mutant (Clontech) gave intensely fluorescent astrocytes that were easily seen in whole-mount preparations (Fig. 5). To our surprise, with GFP as a reporter, the same 2.2 kb promoter region from the human *GFAP* gene that failed to express in Muller cells of the retina and non-myelinating Schwann cells of peripheral nerve now gave consistently detectable signals. Whether this is a question of reporter gene sensitivity to low levels of expression, or unknown interactions of particular reporter genes with specific cell types, remains to be established. Nevertheless, it points out the continued value of reporter gene constructs for fundamental studies of regulation of gene expression and simple cell marking. Such mice should also prove useful for studies of dynamic changes in astrocyte morphology during development, and in response to physiological and pathological conditions.

Having spent considerable time studying or using the promoter for GFAP, we then turned our attention to questions about the role of the GFAP protein itself in astrocyte biology. With the help of Richard Behringer, we generated GFAP-null mice by gene targeting in embryonic stem cells (McCall *et al.*, 1996), an approach simultaneously being taken in three other laboratories (Gomi *et al.*, 1995; Pekny *et al.*, 1995; Liedtke *et al.*, 1996). The results from all four groups indicated that GFAP is not required for overtly normal mouse development. Astrocytes are present in the CNS of the mutant mice, but contain a reduced number of intermediate filaments. Whereas response to some types of injury are normal, such as stab wounds or scrapie infection (Gomi *et al.*, 1995; Pekny *et al.*, 1995), the astrocyte response to inflammatory lesions is impaired (Liedtke *et al.*, 1998). In addition, while early studies of blood brain barrier function suggested that the barrier was intact to large molecules, recent studies suggest that more subtle selective deficits in barrier function might be present (Pekny *et al.*, 1998).

Finally, we and others have founding intriguing evidence for abnormalities in neuron-astrocyte interactions, with GFAP-null mice having impairments in long-term depression in the cerebellum (Shibuki *et al.*, 1996), and enhanced long-term potentiation in the hippocampus (McCall *et al.*, 1996). Curiously, in none of the studies of GFAP-null mice has the morphology of the astrocytes been evaluated in detail, a task made difficult by the absence of GFAP as a marker for the cell and its processes. This is one area in which alternative reporter genes such as the green fluorescent protein should prove especially useful. Overall, the mildness of the phenotype associated with these GFAP mutations was a surprise to many who assumed that this abundant structural protein was critical for astrocyte development and function.

As a complementary approach to studies of GFAP deficiency, we also wished to study the effects of forcing over-expression of GFAP in otherwise normal astrocytes. Increased expression of GFAP is a hallmark of gliosis, the astrocytic hypertrophy that occurs during a wide variety of diseases of the central nervous system (Eng and Ghirnikar, 1994). To determine whether this increase in GFAP expression, *per se*, alters astrocyte function, we generated transgenic mice that carry copies of the human *GFAP* gene driven by its own promoter (Messing *et al.*, 1998). By two weeks after birth the total GFAP content of the brain is elevated several-fold. Astrocytes of these mice become hypertrophic, up-regulate small heat shock proteins, and contain inclusion bodies

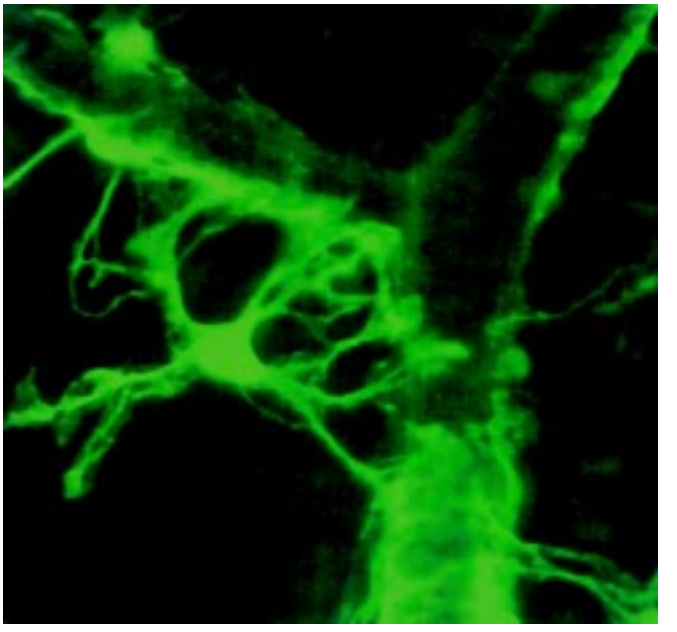

Fig. 5. Living retinal whole-mount from mouse carrying the jellyfish green fluorescent protein under the control of the GFAP promoter (Zhou *et al.*, 1997, Tg94.7 line), visualized by confocal microscopy. *A fluorescent astrocyte is shown with multiple processes in contact with blood vessels.*

identical histologically and antigenically to the Rosenthal fibers of Alexander's disease. Although we do not yet understand how astrocyte function is impaired, widespread deposition of the Rosenthal fibers is associated with early mortality, with mice dying by the second postnatal week. Since we expressed human rather than mouse GFAP, it is important to repeat these experiments to see whether minor sequence differences between the two GFAP's might contribute to the propensity to form inclusions. The GFAP over-expressers also provide intriguing clues to the etiology of Alexander's disease, which we hypothesize may be due to gene duplications or point mutations in the *GFAP* gene. Furthermore, comparison of the phenotypes of GFAP-null mice with the GFAP-over-expressors underscores an increasingly common theme in transgenics and knockouts –too much of something is often much worse than none at all.

Finally, I wish to add a personal note of thanks to Ralph Brinster. I met him when I first applied to the Veterinary Medical Scientist Training Program at the University of Pennsylvania, of which he was the Director for many years. When I traveled to Penn for the interview I promptly got lost in Northeast Philadelphia and then was caught in a torrential downpour, appearing at the doors of the Veterinary School looking like what one member of the admissions committee called "a drowned rat". However, Ralph Brinster has always been an individual of tremendous focus, and he didn't seem to mind the puddles appearing under my chair. For this, and for all the support and guidance he has given me throughout my research career, I am extremely grateful.

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