ABSTRACT

There are many animal models of glioma, but few that represent the biology of low-grade tumors and allow the study of the genetic mechanisms of glial oncogenesis. We report the induction of astrocytic transformation in transgenic mice by the SV40 T antigen under the control of the 5'-flanking sequence of the murine glial fibrillary acidic protein (GFAP) gene. High levels of T antigen expression were detectable in a tissue distribution that mirrored the normal expression of GFAP. This was associated with a consistent phenotype in the founder mice. Diffuse proliferation occurred in cells of the periventricular subependymal zone with diffuse invasion into the brain parenchyma, leading to death by 19–30 days postnatally. Transformed cells exhibited secondary structural, a typical histopathological feature of human astrocytomas. Early passage cultures of these cells expressed GFAP in vitro and were transformed on the basis of tumor formation after transplantation into nude mice. These results demonstrate the susceptibility of periventricular astrocytic cells in the immature brain to malignant transformation. Furthermore, this study demonstrates the potential of the transgenic approach for the in vivo determination of genetic events involved in astrocytic transformation and for the development of novel models of astrocytoma.

INTRODUCTION

The majority of human gliomas are of the astrocytic lineage and may be graded as well-differentiated astrocytoma, anaplastic astrocytoma, and GBM(3, 1, 2). The lower grade tumors typically progress over time to GBM and a lethal outcome (3). Several genetic events that contribute to the malignancy of higher grade gliomas have been identified, but the genetic changes that lead to the formation of lower-grade tumors remain obscure (reviewed in Ref. 4).

Many animal models of glioma have been developed using chemical, viral, and transplantation techniques, but the vast majority of these tumors are highly malignant and rapidly progressive. Several have proved to be useful models of GBM, but very few adequately mirror the biology of lower-grade astrocytomas (reviewed in Refs. 3, 5, and 6). In the era of molecular therapies for glioma (7, 8), there is a need for improved models in which to test such therapies. Such models would also improve the understanding of molecular changes underlying the development and progression of these tumors.

Transgenic models of tumor development using overexpression of oncogenes and disruption of tumor suppressor genes have provided valuable insights into the multiple genetic steps leading to oncogenesis in general (reviewed in Refs. 9–11). The roles of specific oncogenes have been explored in vivo in specific cell types, and their interactions explored by interbreeding different lines of transgenic mice. The neoplastic phase, the stepwise evolution of the oncogenic process, and the effects of potential oncopgenic agents may be studied. Although there is no genetic variation between individual animals in a given transgenic line, the phenotype is typically reproducible over generations. These models have also demonstrated how the process of transformation can differ between tissues (12, 13).

There have been two reports of transgenic induction of malignant gliomas, but these investigations have been incomplete (14) or have been targeted to oligodendrocytes, an uncommon type of human glioma (15). We report the induction of astrocytic transformation in transgenic mice using a chimeric gene expressing the large T Ag of the SV40 virus under the transcriptional regulation of the 5' sequences of the murine gene for GFAP. Expression of GFAP is a key defining characteristic of the astrocyte, although it is not confined to that cell type alone (see "Discussion"). The 5' regulatory region of the murine GFAP gene (GFAPR) has been cloned, sequenced, and characterized in cell culture, where it was able to direct cell-specific expression of a marker gene in primary astrocytes and C6 astrocytic glioma (16–18). To our knowledge, this particular sequence has not been used to regulate gene expression in transgenic mice, although related sequences have been used (see "Discussion").

The T Ag oncogene encodes a protein with multiple cellular activities (reviewed in Ref. 19). Studies involving T Ag mutants in vitro and in transgenic mice have identified activities that appear to be crucial in achieving cellular immortality and/or transformation (20–22). Two such activities are its ability to bind and inactivate the protein products of the tumor suppressor genes, p53 and Rb (reviewed in Refs. 24 and 25). Inactivation of the products of these genes occurs at high and moderate frequency, respectively, in astrocytic gliomas (26, 27). The importance of p53 in glial oncogenesis is confirmed by the elevated frequency of gliomas in the Li-Fraumeni syndrome, which is linked to germline p53 mutation (28, 29). Thus, inactivation of these gene products by T Ag is an appropriate paradigm for a study of astrocytic tumorigenesis.

MATERIALS AND METHODS

Production of GFAPRTAg Transgenic Mice. The recombinant GFAPRTAg fusion gene was constructed using standard techniques (30). Briefly, the GFAPRTAg was excised from the pGF11 plasmid (16) as a 2.3-kb BamHI-XbaI fragment. The T Ag gene was excised from the plasmid pX3 as a 2.7-kb XbaI-BamHI fragment (31). These elements were ligated in a single step reaction into the pBluescript vector (Stratagene), and the configuration was confirmed by restriction mapping. The GFAPRTAg fusion gene was isolated with BamHI, purified, and injected into F2 fertilized eggs of C57BL/6 × DBA mice, which were reimplanted into Swiss outbred mice as described (32, 33). Transgenic mice were identified by Southern blotting of tail DNA, using the above fragment as a probe. The appearance and motor behavior of the mice in these litters were carefully observed every day from the tenth postnatal day.
**Histology and IHC of Mice.** When the mice became lethargic, they were sacrificed using CO₂ inhalation. For two mice, whole brains were immersion-fixed in 10% phosphate-buffered formalin for 3 days, sectioned coronally, and mounted in paraffin. Ten-μm, hematoxylin and eosin-stained coronal sections were examined in conjunction with age-matched normal controls prepared in like manner. For three other mice, the frontal pole of the brain was excised for tissue culture before the majority of the brain was immersion fixed in 4% fresh paraformaldehyde and 1% glutaraldehyde in sodium cacodylate buffer at 4°C for 4 h and examined in like manner. Biopsies of other organs of these three mice and one control mouse were similarly fixed and examined. Tissues examined included the spinal cord, liver, spleen, kidney, heart, lung, pancreas, thyroid, thymus, bladder, skeletal muscle, testis, peripheral nerve, salivary glands, esophagus, stomach, small and large intestine, bone, cartilage, larynx, and pharynx. All histological and immunohistochemical sections were examined in conjunction with an experienced neuropathologist (M. F. G.).

IHC was performed on similar sections using a biotin/streptavidin-alkaline phosphatase technique according to the manufacturer’s instructions (StrAviGen; Biogenex, San Ramon, CA). For the Rb 115 anti-T Ag primary antisera (kindly donated by Dr. D. Lane, Dundee, Scotland), the sections were predigested with 0.05% protease 8 (Sigma Chemical Co.) in PBS at 37°C for 5 min. The primary antisera were diluted to 1:500 for Rb 115 rabbit anti-T Ag serum, 1:500 for rabbit anti-transferrin serum, and 1:150 for rabbit anti-GFAP serum (Dako). The biotinylated secondary antibody was used at 1:100, and the streptavidin-peroxidase reagent at 1:200. All experiments contained sections of normal age-matched mouse brain and other tissues as positive and negative controls. Sections of transgenic mouse brain were used as positive controls when required.

**Tissue Culture.** Cell cultures were established from the brains of the transgenic mice by mechanical dissociation (34) and incubation in 10% FCS in RPMI, with added glutamine (2 mm), penicillin (100 U/ml), streptomycin (100 mg/ml), and fungizone (0.25 mg/ml) all from CSL, Melbourne, Australia) in a humidified 5% CO₂/air incubator at 37°C. Early passage cells were frozen in liquid nitrogen in 10% DMSO in FCS for later experiments.

In some experiments, the cells were grown to over-confluence by maintenance with daily changes of media for 48–72 h after achieving confluence. This method was also used with the addition of X 10⁻¹⁰⁴ m forskolin to the media (35, 36). In separate experiments, the cells were grown in media containing 10 ng/ml BFGF or in low-serum media. This comprised RPMI with 0.5% FCS, 30 μM sodium selenite, 50 μg/ml bovine transferin, and 5 μg/ml bovine insulin (Sigma).

**IIF of Cell Cultures.** For IIF, cultures were grown on sterile coverslips in 24-well plates under conditions as specified above. The coverslips were washed in MT-PBS, then fixed in methanol for 10 min at −20°C. After washing, each coverslip was incubated with the primary antibody diluted in MT-PBS with 0.1% BSA for 30 min at room temperature. After washing, the coverslips were similarly incubated with the appropriate FITC-conjugated secondary antibody. The coverslips were mounted in 4% N-propyl gallate in 90% glycerol/10% PBS, examined with a Nikon fluorescence microscope, and photographed.

The primary antibodies used were rabbit anti-GFAP (Dako) at 1:200 dilution, anti-NFP (M, 150,000; Chemicon) at 1:200, rabbit anti-MBP (Dako) at 1:500, monoclonal anti-T Ag (pAb 419) at 1:16, rabbit anti-T Ag (Rb 115) at 1:150, and monoclonal anti-wild-type p53 at 1:10 (pAb 246, Ab-4; Oncogene Science, Uniondale, NY). The secondary antibodies were swine anti-mouse immunoglobulin, F(ab)₂ fragment, FITC conjugated (Silenus, Hawthorn, Australia) at 1:40 dilution, and swine anti-rabbit immunoglobulin, F(ab)₂ fragment, FITC conjugated (Dako, Copenhagen, Denmark) at the same dilution. For the double labeling, tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit immunoglobulin (Southern Biotechnology, Birmingham, AL) was used at 1:80 dilution.

Double-label IIF was performed using the same method as above, incubating the two antibodies simultaneously on the coverslips. Optimal staining was achieved using the anti-GFAP at 1:200 and anti-T Ag at 1:16 in the first incubation, followed by FITC anti-mouse at 1:80 and tetramethylrhodamine isothiocyanate anti-rabbit at 1:160. To visualize the double-label IIF on individual cells rather than on a population several cells thick, this method was also used on cultures grown to over-confluence in flasks for 2 days, then trypsinized, replated onto coverslips, and allowed to adhere for 12 h before IIF.

Positive controls comprised primary mouse astrocyte cultures for GFAP, a c-myc immortalized cell line NC 14.4.6E (37) for NFP, a neu-immortalized oligodendrocyte cell line for MBP. Negative controls comprised NIH 3T3 cells for GFAP, NFP, MBP, and T Ag, and primary astrocyte cultures for p53.

Electron microscopy was performed according to standard protocols on early passage cultures grown to over-confluence in media containing forskolin. These were harvested by trypsinization, pelleted, washed briefly in MT-PBS, and fixed in 2% glutaraldehyde in sodium cacodylate buffer.

**RNA Analysis of Cell Cultures.** Cells were harvested by trypsinization, and total RNA was extracted (RNAzol; Tel-test, Friendswood, TX). Northern blot analysis was performed using 20 μg total RNA for each sample. Hybridization with random-primed radio-labeled probes (Megaprime; Amersham, Buckinghamshire, United Kingdom) was performed at 65°C in 0.5 μm sodium phosphate buffer pH 7.2, 7% SDS, and 1 mM EDTA (38). The membranes were washed at high stringency using three washes in 0.2 SSC-1% SDS for 20 min at 65°C. The labeled membrane was then incubated with Kodak X-ray film for appropriate periods of up to 10 days. The probes used were the 2.7 kb T Ag fragment, rat c-GFAP (39), the cDNA for rat M, 68,000 neurofilament (40), and murine c-GAPDH (41). Controls included RNA from normal day 14 mouse brain and liver, from cultured NIH 3T3 cells, and from a human colon carcinoma cell line (kindly donated by Dr. R. Whitehead, Ludwig Institute, Melbourne, Australia).

**RESULTS**

**Founder GFAPR/T Ag Mice**

**Phenotype.** Of the 49 live progeny, 11 mice were identified as carrying the transgene by Southern analysis. Nine of these 11 mice exhibited a clinicopathological phenotype of progressive ataxia from postnatal day 14. In seven of the mice, termed “early” mice, this phenotype progressed to lethargy, weight loss, and death at days 18–20. In two mice, the “late” mice, the phenotype was milder and fluctuated in severity until similar progression to death occurred at days 28–30. The remaining two mice were clinicopathologically normal and exhibited no expression of T Ag in the brain. These mice did not transmit the transgene to any progeny and were probably mosaic. No correlation between the phenotype of the animals and the transgene copy number, as assessed by the Southern analysis, was observed.

**Histology and T Ag Expression.** Three “early” and 2 “late” mice were autopsied in detail. Gross examination of the mice was unremarkable, except that there was marked hydrocephalus in the two “late” mice. Histological examination of all five brains revealed a similar-appearing atypical cellularity, clustering around blood vessels and neurons. Similar-appearing atypical cells were scattered throughout the brain. They exhibited secondary structuring similar to that seen in human astrocytic tumors, clustering around blood vessels and neurons and accumulating in the subpial compartment. Mitoses were also seen amongst the atypical cells in the cortex. In the age-matched normal controls, no mitoses were seen in the brain. Two small, well-defined focal tumors were found in one of the brains, but the process was otherwise diffuse (Fig. 1). The brains appeared to be well formed, although there was some distortion of the regular neuronal layers of the hippocampus and the convexity cortex. The choroid plexus in the “early” mice appeared slightly hyperplastic as compared to the normal mice, but the ventricles appeared normal.

In the two “late” mice, the abnormal cellularity was less marked in the periventricular region. The distribution of atypical cells in the cortex was similar to that observed in the “early” mice, although less...
Fig. 1. The histological appearance of the brains of "early" GFAPR/T Ag mice (b, d, f, and h) as compared to similar regions of the brains of normal age-matched mice of the same genetic background (a, c, e, and g). The periventricular region on the lateral aspect of the lateral ventricle (a and b); X 160. The small atypical cells with dark nuclei accumulate in the subependymal region and exhibit secondary structuring in the rest of the brain. Hippocampus (c and d); X 40. Note the distortion of normal neuronal architecture. Cerebral cortex of the convexity of the hemisphere, X 400 (e) and X 160 (f). Note the secondary structuring of atypical cells around neurons and in the subpial plane. Choroid plexus of a control mouse (g), X 400, and a “late” GFAPR/T Ag mouse (h), X 160.

dense in some regions. These brains exhibited marked diffuse hyperplasia of the choroid plexus of all four ventricles, which was the probable cause of their hydrocephalus and death (Fig. 1). No focal tumors were seen in the choroid plexus. The other tissues of these five mice appeared identical to age-matched normal control tissues (data not shown).

Immunohistochemistry. The atypical astrocytic cells exhibited specific nuclear immunoreactivity for T Ag in all five brains examined (Fig. 2). There was no such reactivity in the control brains. This reactivity was less intense in the cells immediately adjacent to the ventricles than in the atypical cells elsewhere in the brain. All the cells in the focal tumors found in one of these brains exhibited very strong immunoreactivity. There was also moderately strong nuclear T Ag expression in virtually all choroid epithelial cells. This uniformity, the morphology, and the positive immunoreactivity for transthyretin in the hyperplastic choroid plexus (described below) indicate that the T Ag was expressed in the choroid epithelium, rather than the choroid being invaded by T Ag-positive cells from the periventricular zone. There was no T Ag reactivity in neurons at any site in the CNS or peripheral nervous system.

In the spinal cord and brain-stem, there were a modest number of T Ag-positive cells scattered throughout otherwise normal-appearing tissue (Fig. 3). No focal tumors were seen. There was T Ag expression in a moderate proportion of the nuclei in the spinal nerve roots, dorsal root ganglia, and spinal and peripheral nerves. On morphological grounds, these cells appeared to be Schwann cells in the nerves and satellite cells in the dorsal root ganglia.

In the esophagus, stomach, and small and large intestine, there was strong nuclear T Ag expression in cells corresponding anatomically to the myenteric, submucosal, and even mucosal plexes of the enteric nervous system, suggesting expression in enteric glia (Fig. 3). In the pancreas, there were clusters of positive nuclei in and around the islets of Langerhans, consistent with expression in glia of the pancreatic ganglia, a part of the enteric nervous system (42).

Small clusters of T Ag-positive nuclei were seen in sites corresponding to plexes and nerves of the autonomic nervous system, such as in the adventitia of blood vessels in several organs, the capsule of the spleen, and adjacent to large bronchi and tubules of the epididymis. On morphological grounds, this was consistent with expression in non-myelinating Schwann cells. There was nuclear expression of T
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Fig. 2. T Ag expression in the brain of the GFAP/T Ag (b, d, e, and f) and control (a and c) mice as demonstrated by IHC. T Ag-positive cells exhibit dark nuclei due to 3,3'-dimethylaminoazobenzene accumulation, whereas negative cells exhibit pale nuclei due to dilute hematoxylin counter-stain. Lateral ventricle showing periventricular zone and choroid plexus (a and b); × 100. Cerebral cortex of the convexity of the hemisphere (c and d); × 160. The T Ag-positive cells exhibit secondary structuring around neurons. Note the more intense staining in these cells as compared to the cells in the periventricular zone. Well-defined and poorly defined focal tumors in the brain of one "early" mouse (e and f); × 100.

Ag in chondrocytes in multiple sites and in myoepithelial cells in salivary glands of the pharynx and larynx. There was strong T Ag expression in nuclei of the transitional epithelium of the urinary bladder. The level of T Ag expression appeared to be subjectively similar at all these sites, as determined by IHC.

The five founder brains were examined for GFAP expression by IHC. The atypical cells in the periventricular zone in these brains exhibited positive cytoplasmic reactivity for GFAP, suggesting that these were of astrocytic lineage, but this was not observed in the atypical cells in the rest of the brain (data not shown). The core of the hypertrophic choroid plexus stained very weakly for GFAP but not the superficial cells. Normal GFAP-positive astrocytes with typical arborized processes and normal-looking nuclei were seen in the hippocampus and other areas of the brains. These brains were also examined by IHC for the presence of transthyretin, a protein specifically synthesized in the choroid plexus epithelium and liver (43, 44). The hypertrophic choroid was clearly positive for this antigen, particularly in the superficial layers, whereas there was no staining of any cells in the brain parenchyma.

Cell Lines

Cell cultures were successfully established from two of the three brains of GFAP/T Ag mice in which this was attempted. Of these two cultures, one (GT-A) was derived from an "early" mouse and the other (GT-B) from a "late" mouse. The cells in these two cultures grew rapidly from the outset and were characterized at early (4–5 passages) and late passage (30–35 passages). These cultures were maintained in culture for 6 months, or approximately 50 passages. There was never any period of slow growth or crisis. The doubling time of the early passage cultures in the exponential phase was approximately 17 h and that of the late passage cultures, approximately 11 h (data not shown). Both early and late cultures exhibited a high degree of serum dependence when plated at low densities but not at high densities. Single cell cloning of the early passage cultures was attempted in media containing 10% FCS but was unsuccessful.

By light microscopy, different subpopulations of cells were apparent in both of the early passage cultures (Fig. 4). A small proportion of cells developed broad irregular sheets of cytoplasm with multiple fine processes, similar to primary astrocytes, although they did not express detectable GFAP. The majority of cells exhibited an epithelioid appearance with a polygonal outline and multiple short processes. Other cells exhibited an undifferentiated appearance, with a smaller, triangular shape and short processes. The growth of the cells was not contact inhibited, and they formed spontaneous foci of closely packed spindle-shaped cells. After about 8–10 passages, the largest phenotype was no longer seen in the cultures. With further passage, the cells gradually became uniform in appearance, becoming smaller with an increased nuclear:cytoplasmic ratio and a more stellate or triangular outline (Fig. 4).

Both early and late passages of both cultures were tumorigenic after injection of 10⁶ cells intracranially or s.c. into nude mice. Interestingly, these tumors were very slowly growing with a latency of 12–20 weeks and exhibited diffuse spread of single T Ag-positive cells throughout both hemispheres of the brain, with secondary structuring around neurons and blood vessels.

Identification of Cell Lineage Markers. IIF of both cell cultures at both early and late passage confirmed nuclear staining for T Ag in all cells using monoclonal and polyclonal antisera (Fig. 4). Thus, the cells in culture were derived from the atypical cells in the founder brains. There was also strong nuclear staining for wild-type p53 in all.

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5 R. A. Danks, unpublished observations.
Fig. 1. T Ag expression in the spinal cord and peripheral nerves and enteric plexus of transgenic (h, d, f, and h) and control mice (a, c, e, and g). Spinal cord, × 40 (a and b) and × 100 (c and d). Scattered positive nuclear staining in nonneuronal cells in the cord, dorsal root ganglion, and spinal nerve. Dorsal root ganglion and proximal spinal nerve (e and f), × 100. Intestine (g and h), × 100 and × 40. This distribution is consistent with expression in cells of the enteric plexus of the gut.

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four lines but not in control cultures. This confirmed the expected accumulation of p53 protein due to sequestration by T Ag.

In nonconfluent cultures, there was no detectable GFAP expression, but early passage cultures of both cell lines exhibited coarse fibrillary cytoplasmic staining for GFAP in a moderate proportion of the cells when the cultures were grown for 2–3 days after confluence (Fig. 4). This staining was more pronounced when 3 × 10^{-6} M forskolin was added to the cultures. This agent causes accumulation of cAMP in the cells and promotes differentiation including increased GFAP expression in cultured astrocytes and malignant astrocytic lines (45–48). Double-label IIF of these early cultures demonstrated that cytoplasmic staining for GFAP and nuclear staining for T Ag occurred in the same cells (data not shown).

The cultures were also subjected to IIF for M, 150,000 NFP. They did not express this marker of neuronal differentiation when grown either with or without added bFGF (data not shown). bFGF encourages neuronal differentiation, including expression of NFP in primary cultures of primitive neuroepithelial cells (49) and in bipotential transformed lines (50–52). Similarly, there was no detectable expression of MBP when the cultures were grown either in standard media or in low-serum media (data not shown). Growth in low-serum media encourages the expression of oligodendrocytic markers in precursor cells or bipotential lines (15, 53).

These results were confirmed by RNA analysis (Fig. 5). By Northern blotting, GFAP mRNA expression was detectable as a 2.7-kb band in RNA from both early passage cultures grown to overconfluence. Two different-sized T Ag transcripts were detectable in both early and late passage cultures. There was no detectable expression of the mRNA for the M, 68,000 NFP in any of the cultures.

Electron microscopy was performed upon early passage cultures grown to over-confluence in the presence of forskolin. No features typical of any neuroepithelial lineage were visible on electron microscopy. In particular, no intermediate filaments or intercellular adhe-
Fig. 4. Cell cultures. Phase contrast photographs (× 500) of the early (GT-AE; A) and late passage cultures (GT-AL; B) from the brain of an "early" phenotype mouse. Early passage means 4–5 passages from initial culture, and late means 30–35 passages. C-H, IIF of the cell cultures; × 500. Strong nuclear IIF was seen using anti-T Ag and anti-p53 (pAb 246) anti-sera on GT cultures but not in control NIH 3T3 cultures. C, GT-AE, anti-T Ag; D, NIH3T3, anti-T Ag, negative control; E, GT-AE, anti-p53. IIF with anti-GFAP anti-serum of primary mouse astrocytes as a positive control (F); NIH 3T3, negative control (G); and GT-AE (H). These latter cultures (F and G) were grown to over-confluence with added forskolin, as described. This causes many of the cells to be out of focus, because there were several layers of cells in these cultures, rather than a monolayer culture. Note the fibrillary expression of GFAP in the GT-AE cultures, although this was always less dense than in the primary astrocytes.

sions were visible. Cytogenetic analysis was performed on early and late passages of the GT-A culture. Chromosomes 4 and 5 were consistently trisomic. Inconsistent trisomies and tetrasyomies were observed for other chromosomes, resulting in a high degree of heterogeneity, which could result from genetic instability caused by T Ag.

DISCUSSION

These investigations involve the application of transgenic methodology to the study of astrocyte transformation. The GFAP/T Ag mice exhibited an early and lethal proliferation of cells of the periventricular subependymal zone of the immature brain, associated with strong expression of the transgene. In vivo, the astrocytic nature of these T Ag-transformed cells was demonstrable by their GFAP expression and by their manner of spread within the brain. They exhibited a uniform cellular morphology and diffuse invasion and secondary structuring around neurons and blood vessels, which is characteristic of human astrocytomas. Both these features were also clearly evident on transplantation of the two early passage cultures into the brains of mature nude mice.

In vitro, GFAP expression was confirmed in early passage cultures at both the protein and RNA levels, but only when the cultures were grown to over-confluence. This expression was unlikely to be due to reactive nonneoplastic astrocytes for two reasons: (a) no T Ag-negative cells were seen in the cultures; and (b) no GFAP expression was seen in nonconfluent cultures of these cells. Even in the overconfluent cultures, the level of GFAP expression was low, and no intermediate filaments were demonstrable on electron microscopy.
This lack of intermediate filament formation has been reported previously with GFAP-positive cell lines, including C6 (54, 55), and was felt to represent abnormal posttranslational assembly of the protein in vitro (56). In the GT cultures, this may be due to suppression of GFAP expression and assembly by the effects of the T Ag. Such an effect was observed in primary mouse cerebellar cultures transfected with temperature-sensitive T Ag, when those lines that expressed GFAP strongly at the nonpermissive temperature lost such expression after changing to the permissive temperature (57). Alternatively, the low level of GFAP expression in the T Ag-transformed cells may reflect their transformation at an immature stage.

**Pattern of Transgene Expression.** The proximal 2.3 kb of the 5′-flanking sequence of the GFAP gene (GFAPR) was able to direct strong cell-specific expression of the T Ag in a pattern that corresponded to the normal pattern of GFAP expression, with few aberrations. In the normal mouse, GFAP expression has been described in CNS astrocytes (58, 59), satellite cells of dorsal root and sympathetic ganglia, non-myelinating Schwann cells of peripheral nerves (60-62), enteric glia (63), chondrocytes (64, 65), olfactory epithelium (66), myoepithelial cells of salivary and lacrimal glands (67-69), and ocular lens epithelium (70). In the GFAPR/T Ag mice, strong and specific T Ag expression was found in all of these cell types that were examined.

However, there were three examples of possible aberrant expression, possibly reflecting the fact that not all of the regulatory elements of the GFAP gene were present in the sequence used in the GFAPR/T Ag mice: (a) strong T Ag expression throughout the choroid plexus was a major problem in this experiment. In the “two” late mice, the demise of the animals appeared to be mainly due to hydrocephalus secondary to choroid plexus hyperplasia, rather than astrocytic proliferation, which was less marked in these animals. Normally, GFAP expression is undetectable in the choroid plexus of rodent and human brains (71-74), although it was detectable in a defined portion of the choroid plexus, the “Ependymzotten,” in one study of neonatal human brains (74). Interestingly, 40-50% of human choroid plexus papillomas exhibit areas of strong GFAP expression, which may reflect this developmental expression (71-73); (b) a less important aspect of possible aberrant expression was the strong nuclear T Ag expression observed in the transitional epithelium of the urinary bladder of the GFAPR/T Ag mice, a cell type where specific cytokeratins are expressed (75) but where GFAP expression has not been assessed; and (c) the actual level of expression of the T Ag protein appeared to be similar in all of the cells where it was expressed, as determined by IHC. This may also represent an aberration from normal patterns of expression, because the levels of normal GFAP expression are higher in CNS astrocytes than in enteric glia and are perhaps 20-fold lower in non-myelinating Schwann cells (60).

This pattern of expression can be contrasted with the patterns of expression of chimeric transgenes controlled by the entire murine GFAP gene with its flanking sequences and those controlled by the 5′-flanking sequence of the human GFAP gene (76-79). The transgenic mice expressing genes under the control of these sequences exhibited astrocyte-specific gene expression at a low level. This
expression was inducible by brain injury, reproducing the normal strong induction of GFAP expression in reactive astrocytosis. Peripheral expression was absent or limited. These results are consistent with the presence of important negative regulatory elements within the GFAP gene downstream of the transcriptional start-point, which have been demonstrated by in vitro analysis (18).

Transformation of Cells in the Periventricular Germinal Matrix. Compared with previously reported transgenic mice expressing T Ag in a wide variety of cell types (reviewed in Refs. 9 and 10), the TAg-expressing astrocytic cells in the brains of the GFAPR/T Ag mouse have undergone unusually rapid transformation before 20 to 30 days after birth. The cells of the choroid plexus exhibited obvious hyperplasia, whereas the other cell types expressing T Ag may have undergone limited hyperplasia or not responded to it, despite the presence of similar levels of T Ag immunoreactivity. This may be due to different temporal patterns of T Ag expression in these populations during development. Alternatively, the temporal pattern of expression may reflect the normal developmental patterns of GFAP expression. In normal mice, GFAP mRNA transcripts are detectable in the brain, enteric nervous system, and peripheral nerves from around embryonic days 16–18. The level of expression increases dramatically over the first two postnatal weeks (59, 80–82). The gradual rise of GFAP expression in the hindbrain as determined by in situ hybridization precedes the rise in the cerebrum by about 5 days (83). Thus, it might have been expected that T Ag expression would have occurred slightly later in the cells of the periventricular region than at other sites, whereas the most vigorous proliferation and transformation occurred in the periventricular region.

This rapid transformation of the astrocytic cells in the periventricular region of the GFAPR/T Ag mice is consistent with previous demonstrations of the exquisite sensitivity of these cells to transforming influences, as previously shown in chemical and viral models of glioma (reviewed in Refs. 3 and 84) and by transfection with the v-src oncogene (85). This susceptibility may be due to the fact that the periventricular subependymal zone is the normal germinal zone for the telencephalon and that there is a rapid developmental proliferation of immature cells in this region immediately postnatally (86–88). Some of these cells start to express GFAP at this time. Thus, the T Ag expression may have coincided with this developmental proliferation in the GFAPR/T Ag mice. This may have rendered the cells more susceptible than usual to transformation.

In conclusion, this work demonstrates that transgenic techniques may be systematically applied to the study of astrocytic oncogenesis and to a continuous evaluation of better animal models of glioma. Furthermore, this demonstrates the availability of a suitable promoter system using the 5′-flanking sequence of the GFAP gene to express a chosen gene at high levels in the astrocytes of transgenic mice, as well as in glia in the peripheral nervous system and other specific cell types. This contrasts with the lower level expression of chimeric genes in CNS astrocytes alone in transgenic mice regulated by the entire GFAP gene. This also confirms the susceptibility of immature astrocytes in the developing murine brain to malignant transformation by a single exogenous stimulus.

Future transgenic experiments to study astrocytic oncogenesis may be usefully based upon the same promoter sequence used in this study (GFAPR) but using a less potent oncogene than T Ag.

Alternatively, the entire murine GFAP gene may be an appropriate promoter. This would allow a degree of experimental control over the timing of oncogene expression, because basal expression of the transgene was low with that promoter but could be induced by brain injury.

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Hanahan, D. The subependymal layer of the mouse brain and its cell production as shown on April 4, 2017. © 1995 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from


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