



# Reexpression of Glial Fibrillary Acidic Protein Rescues the Ability of Astrocytoma Cells to Form Processes in Response to Neurons

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**Abstract.** Astroglial cells play an important role in orchestrating the migration and positioning of neurons during central nervous system development. Primary astroglia, as well as astrocytoma cells will extend long stable processes when co-cultured with granule neurons. In order to determine the function of the glial fibrillary acidic protein (GFAP), the major intermediate filament protein in astroglia and astrocytoma cells, we suppressed the expression of GFAP by stable transfection of an anti-sense GFAP construct in human astrocytoma U251MG cells. The resulting AS2-U251 cells can no longer extend stable processes in the

presence of granule neurons. To show that this effect is due specifically to the absence of GFAP, we reintroduced a fully encoding rat brain GFAP cDNA into these AS2-U251 cells. The resulting rat GFAP appeared as a filamentous network and the reexpression of GFAP **rescued** the ability of these astrocytoma cells to form stable processes when co-cultured with neurons. From these results, it is clear that the glial specific intermediate filament protein, GFAP, is required for process extension of these astrocytoma cells **in response to granule neurons.**

**N**EURON-GLIA interactions are important for neuronal migration (Rakic, 1971; Rakic and Sidman, 1973; Sidman and Rakic, 1973; Levitt and Rakic, 1980), neurite outgrowth (Tomaselli et al., 1988), and axon guidance (Silver et al., 1982) in the developing mammalian brain. In the mouse cerebellum, postmitotic granule cells leave the external granule cell layer during the first two postnatal weeks and descend into the internal granule cell layer by migrating along the fibers of Bergmann glia (Sidman and Rakic, 1973; Rakic and Sidman, 1973; Rakic, 1971; Gao and Hatten, 1993). By using an in vitro microwell culture system, Hatten and co-workers showed that, in the presence of granule cells, astroglial cells differentiate into elongated, radial forms which resemble the glial forms that have been shown to support neuronal migration in brain (Hatten et al., 1984; Hatten, 1985, 1987; Mason et al., 1988). Granule neurons can be demonstrated to move along these glial processes in vitro (Hatten, 1990; Edmondson and Hatten, 1987; Hatten and Mason, 1990) with a morphology similar to those described in vivo (Rakic, 1971; Rakic et al., 1974). The neurons tightly appose their soma along the glial processes and extend a thickened leading process in the direction of migration (Edmondson and Hatten, 1987; Hatten and Mason, 1990). Similar migratory behaviors have also been demonstrated with both large and small hippocam-

pal neurons (Gasser and Hatten, 1990a). In vivo studies on axonal guidance in the optic nerve (Bovolenta et al., 1987; Williams and Rakic, 1985) and in the white matter of the cerebellum (Pinto-Lord et al., 1982) also demonstrated that astroglia intercalate with growing axons. The navigational instruction for migration seems to be encoded in the neurons, not astroglia, since neurons from different brain regions migrate freely on heterotypic astroglial cells. The mode of migration, neuron-glia relationship, and dynamics and speed of movement of the neurons show no demonstrable differences (Gasser and Hatten, 1990b).

Like neuronal processes, astrocytic processes are tipped by a growth cone-like structure (Mason et al., 1988). However, whereas the neuronal growth cones extend in the absence of cell-cell contacts, true glial process extension (in contrast to cavitation of flattened processes) appears to depend on the formation of cell-cell contacts with neurons (Mason et al., 1988; Hatten, 1985, 1987; Hatten et al., 1986; Edmondson et al., 1988; Weinstein et al., 1990; Torres-Aleman et al., 1992). Another distinction between growing glial and neuronal processes is the abundance of intermediate filaments in the extending glial processes, and their relative paucity in neuronal processes (Mason et al., 1988). Glial fibrillary acidic protein (GFAP),<sup>1</sup> the major protein constituent of glial intermediate filaments, is found in the

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1. *Abbreviations used in this paper:* CM-PBS, calcium- and magnesium-free PBS; IF, intermediate filament; GFAP, glial fibrillary acidic protein; MAP4, microtubule-associated protein 4.

central core of glial processes by both immunostaining and immunoelectron microscopy.

To determine if GFAP is central to any of these changes, we previously suppressed the expression of GFAP in U251MG cells, a permanent human astrocytoma cell line (Westermarck, 1973), by transfection with an expression construct constitutively transcribing the anti-sense strand of a murine GFAP cDNA (Weinstein et al., 1991). The results showed that, with the suppression of GFAP expression, these AS2-U251MG cells were unable to extend complex processes in response to neurons. Other cytoskeletal components such as vimentin and the microtubule-associated protein, MAP4, were not affected and formed normal filamentous networks. These results suggested that GFAP is required for glial process formation in the presence of neurons.

In this study, we have isolated a fully encoding rat GFAP cDNA clone and used it to reexpress GFAP in the GFAP-suppressed, AS2-U251 cells (Weinstein et al., 1991). Our results show that, after reexpression of GFAP, these cells regain the ability to extend processes when co-cultured with granule neurons.

## Materials and Methods

### Antibodies

Polyclonal anti-GFAP antibody (pAb-GFAP) is a rabbit antiserum (Wang et al., 1984), used at a dilution of 1:200. G-A-5, a monoclonal anti-GFAP antibody used in double labeling experiments at a dilution of 1:250, was obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-microtubule-associated protein 4 antiserum, LHB (pAb-MAP4), a kind gift of Dr. J. C. Bulinski (Columbia University, New York), is a polyclonal rabbit antiserum that specifically recognizes primate MAP4 (Bulinski and Borisy, 1980). This anti-MAP4 antiserum allowed us to stain only the human astrocytoma cells without interference from the small number of rat astrocytes contaminating our preparations of neurons. Goat anti-rabbit and anti-mouse IgG's conjugated to either FITC or TRITC were purchased from Organon Teknika Corp. (Charlotte, NC).

### Screening of Rat Brain cDNA Library

A 2-kb fragment of murine GFAP cDNA (Lewis et al., 1984), a kind gift from Dr. N. J. Cowan (Department of Biochemistry, New York University, New York), was used to screen an amplified rat brain  $\lambda$ -gt11 cDNA expression library (generously provided by Dr. D. Colman, Mt. Sinai University, New York), following procedures described previously (Napolitano et al., 1987; Chin and Liem, 1990).

### In Vitro Transcription and Translation

The GFAP cDNA-containing clone G92 was digested with EcoRI and the resulting insert fragment was subcloned into pGEM-7Zf (Promega Biotec, Madison, WI), which had been previously digested with EcoRI and treated with calf intestine phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). In vitro-synthesized transcripts of the resulting plasmid, pGEM7Zf-G92, were generated by either T7 or SP6 RNA polymerases according to the protocol suggested by Riboprobe Gemini System (Melton et al., 1984). Briefly, 5  $\mu$ g of plasmid was mixed with appropriate concentrations of transcription buffer, DTT, CAP, RNasin, and either T7 or SP6 polymerase and incubated in 37°C for 1 h. These RNA transcripts were then translated in a rabbit reticulocyte lysate system (New England Nuclear, Wilmington, DE) following the protocol recommended by the manufacturer. In brief, the RNA transcripts were mixed with [<sup>35</sup>S]methionine, potassium acetate, magnesium acetate, and translation cocktail provided by manufacturer, and incubated in 37°C for 1 h. The resulting product was separated by SDS-PAGE followed by autoradiography.

### DNA Sequencing

The GFAP cDNA clone (G92) was subcloned, in both orientations, into the

unique EcoRI site of bacteriophage M13mp18. Sequentially shorter, overlapping subclones in both orientations were created by the rapid deletion method developed by Dale et al. (1985), using T4 DNA polymerase purchased from Promega Biotec. The terminal deoxyribonucleotidyl transferase and subcloning primer, RD29-mer, were purchased from IBI-A Kodak Co. (New Haven, CT). DNA sequencing was performed according to the dideoxy chain termination sequencing protocol by Sanger et al. (1980) but using Sequenase v.2.0 (UBS, Cleveland, OH) and deoxyadenosine 5'-( $\alpha$ -<sup>35</sup>S-thio) triphosphate (New England Nuclear).

### Sequence Analysis

Nucleotide and amino acid sequences were analyzed using the DNA/Protein Sequence Analysis System software developed by James M. Pustell and IBI (Pustell and Kafatos, 1984).

### Construction of GFAP Expression Vectors

The 2.7-kb rat GFAP cDNA insert was isolated by digestion of the pGEM7Zf-G92 plasmid with EcoRI. After gel purification, this fragment was blunt-ended with the large Klenow fragment of *Escherichia coli* DNA polymerase I and ligated with HindIII linkers (Boehringer Mannheim Biochemicals) freshly phosphorylated by T4 polynucleotide kinase. After digestion with excess HindIII and gel purification, the GFAP-encoding fragment was cloned into the unique HindIII site of the expression vectors pSV2i-HindIII and pRSVi-HindIII (generously provided by Drs. B. Forman and H. Samuels, New York University Medical Center, New York). The resulting expression vectors, pSV2i-GFA and pRSVi-GFA, were amplified in HBI01.

### Cell Culture

Mouse L tk<sup>-</sup> cells were cultured in DME containing 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin sulfate (GIBCO BRL, Gaithersburg, MD) and maintained in a 37°C incubator under 7% CO<sub>2</sub> and 80% humidity. Human astrocytoma cell line, U251MG, a kind gift of Dr. B. Westermarck (University of Uppsala, Uppsala, Sweden) was cultured in DME containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin sulfate, 1% non-essential amino acid and maintained in a 37°C incubator under 7% CO<sub>2</sub> and 80% humidity. AS2-U251 cells whose endogenous GFAP had been suppressed by constitutive expression of the anti-sense strand of murine GFAP cDNA (Weinstein et al., 1991) were cultured under the same conditions as the parent cell line, U251MG, except that 500  $\mu$ g/ml G418 (Geneticin, GIBCO BRL) was added to their media. In the case of the **rescue** experiments in which AS2-U251 cells were stably co-transfected with GFAP-expression vectors and the Hygromycin-resistance gene, 300  $\mu$ g/ml Hygromycin B (Sigma Chemical Co., St. Louis, MO) was added in addition to 500  $\mu$ g/ml G418.

### Transient Transfections

The transient transfection protocol using lipofectin (GIBCO BRL) was modified from that described by Felgner and Ringold (1989). Briefly, cells were grown to 70–80% confluence in 35-mm tissue culture plates and washed twice with calcium- and magnesium-free PBS (CM-PBS), and once with serum-free media. 0.5 ml of a lipofectin-DNA mixture, consisting of a mixture of 5–10  $\mu$ g pRSVi-GFA DNA or pSV2i-GFA DNA and 5–15  $\mu$ g of lipofectin diluted in 0.5 ml of serum-free culture medium, was then added to each plate. After incubation at 37°C for 3–5 h, an additional 0.5 ml of serum-free medium was added to each plate. The medium was replaced 6–12 h later with 2 ml culture medium containing 10% FBS, and aliquots of the cells were examined during the next 24–72 h.

### Stable Transfections

Stable transfections were performed using the same procedures described for the transient transfections except that, after the cultures became confluent in 35 mm plates, they were split onto two 100 mm tissue culture plates and grown in the appropriate selection medium (i.e., G418 for L tk<sup>-</sup> cells and G418 plus Hygromycin B for the stable transfections of AS2-U251 cells). The corresponding antibiotic resistance genes, pSV2-Neo and pUC-Hyg 1.1, were co-transfected with either pRSVi-GFA or pSV2i-GFA in a 1:10–20 (wt/wt) ratio. The pUC-Hyg 1.1 vector is a kind gift from Dr. T. M. DeChiara (Columbia University).

## Immunofluorescence

Unless otherwise specified, all immunocytochemical experiments were performed in Lab-Tek eight-well tissue culture slides (Miles Laboratories, Inc., Naperville, IL) coated with 50 ng/ml poly-L-lysine (Sigma Chemical Co.). About  $1 \times 10^4$  cells suspended in 0.2–0.4 ml of culture medium were added to each well and incubated at 37°C in 7% CO<sub>2</sub> at controlled humidity for 24–48 h. Cells were fixed in ice cold methanol for 7 min and then washed with CM-PBS. Nonspecific binding was blocked by incubation with 5% FBS, diluted in CM-PBS, for 30 min at room temperature. The blocking solution was replaced with polyclonal rabbit anti-GFAP antibody, diluted 1:200 in CM-PBS, and incubated for 1 h at room temperature. After washing with CM-PBS and incubating with fluorescein (FITC)-conjugated or rhodamine (TRITC)-conjugated goat anti-rabbit immunoglobulin, diluted 1:300 in CM-PBS, for 20 min at room temperature, the slides were mounted in a mixture of 16% polyvinyl alcohol (Sigma Chemical Co.) and 33% glycerol in CM-PBS, and examined with a Nikon Optiphot microscope equipped for epifluorescence. In the case of co-culture experiments, the same immunostaining protocol was followed except that pAb-MAP4 and G-A-5 were used as primary antibodies and FITC-labeled goat anti-mouse IgG and TRITC-labeled goat anti-rabbit IgG antiserum as secondary antibodies. In experiments where the length and number of the processes were measured, we recorded the immunostained cultures with a Silicon Intensified Target Camera (65 series; Dage-MTI, Inc., Michigan City, IN) and digitized them with an Image-1 image analysis program (Universal Image Corp., West Chester, PA).

## Western Blot Analysis

Preparation of cytoskeletal proteins and Western blotting were performed as described (Ching and Liem, 1993), except that 10% SDS-PAGE at a pH of 9.2 was used to enhance the mobility difference between human and rat GFAP. Monoclonal anti-GFAP antibody, G-A-5, diluted 1:400 in PBS was used as primary antibody and <sup>125</sup>I-labeled goat anti-mouse IgG (New England Nuclear) was used as secondary antibody. The blot was exposed with intensifying screen for 8 h at –80°C using Kodak XAR-5 Scientific Imaging Film. Densitometric scanning was done using an Apple OneScanner with Ofoto scanning software (Apple Comp. Inc., Cupertino, CA) and analyzed by NIH Image 1.43 Gel Analysis program (National Institutes of Health, Bethesda, MD).

## RNase Protection Analysis

Total cellular RNA was isolated following the protocol described by Chomczynski et al. (1987). pGEM7Zf-G92 was digested with ApaI and religated to form pGEM7Zf-G92(ApaI) which included the entire protein coding region and 12 nucleotides beyond the termination codon. Antisense riboprobes of rat GFAP were transcribed by T7 polymerase using pGEM7Zf-G92(ApaI) as template and labeled with [ $\alpha$ -<sup>32</sup>P]CTP following the protocol described by Melton et al. (1984). The entire coding region of the human GFAP was synthesized by PCR (Chen and Liem, 1994) and cloned into the unique HindIII site of pGEM3Z. The resulting plasmid pGEM3Z-hGF was transcribed by SP6 polymerase to make the antisense riboprobe of human GFAP. RNase protection analyses were modified from protocols described by Ching and Liem (1991). Briefly, 20  $\mu$ g of total RNA was hybridized with  $5 \times 10^5$  cpm of appropriate riboprobes in 30  $\mu$ l of hybridization buffer (80% formamide, 40 mM Pipes (pH 6.7), 0.4 M NaCl, and 1 mM EDTA). After incubations at 85°C for 5 min and subsequently at 45°C overnight, 300  $\mu$ l of RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 200 mM sodium acetate) plus 15 U RNase ONE (Promega Biotec) was added to the hybridization mixture and incubated at 30°C for 60 min. The activity of RNase ONE was irreversibly inactivated by the addition of 5  $\mu$ l of 10% SDS containing 4  $\mu$ g/ $\mu$ l of carrier tRNA. The protected RNAs were isolated by ethanol precipitation and subsequently resuspended in 10  $\mu$ l of loading buffer (80% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, and 0.1% SDS). The samples were resolved on a 6% polyacrylamide/8 M urea gel. Autoradiography was performed by exposing the gel to Kodak XAR-5 film at –70°C with an enhancing screen.

## Co-culture Experiment

Co-culture experiments with postnatal day 3 to day 5 rat cerebellar granule neurons were performed as described by Weinstein et al. (1991) except that neurons were added to astrocytic cells in a ratio of 60–100:1 to saturate potentially responsive cells.

## Process Measurement

A process was defined as any protrusion with a tipped end and a base whose width was shorter than the length of the protruding part. The length of the entire process, however, was defined as the distance from the geometric center of the nucleus of the cell to the very end of process. At least 55 cells were systematically picked for measurement of process length and number. The results were expressed as average process number and length per cell. To minimize the effect of variability between cell lines, the percentages of increase or decrease of process length and number in the presence and absence of neurons were determined separately for each cell line.

## Results

### Characterization of a Fully Encoding Rat GFAP cDNA Clone

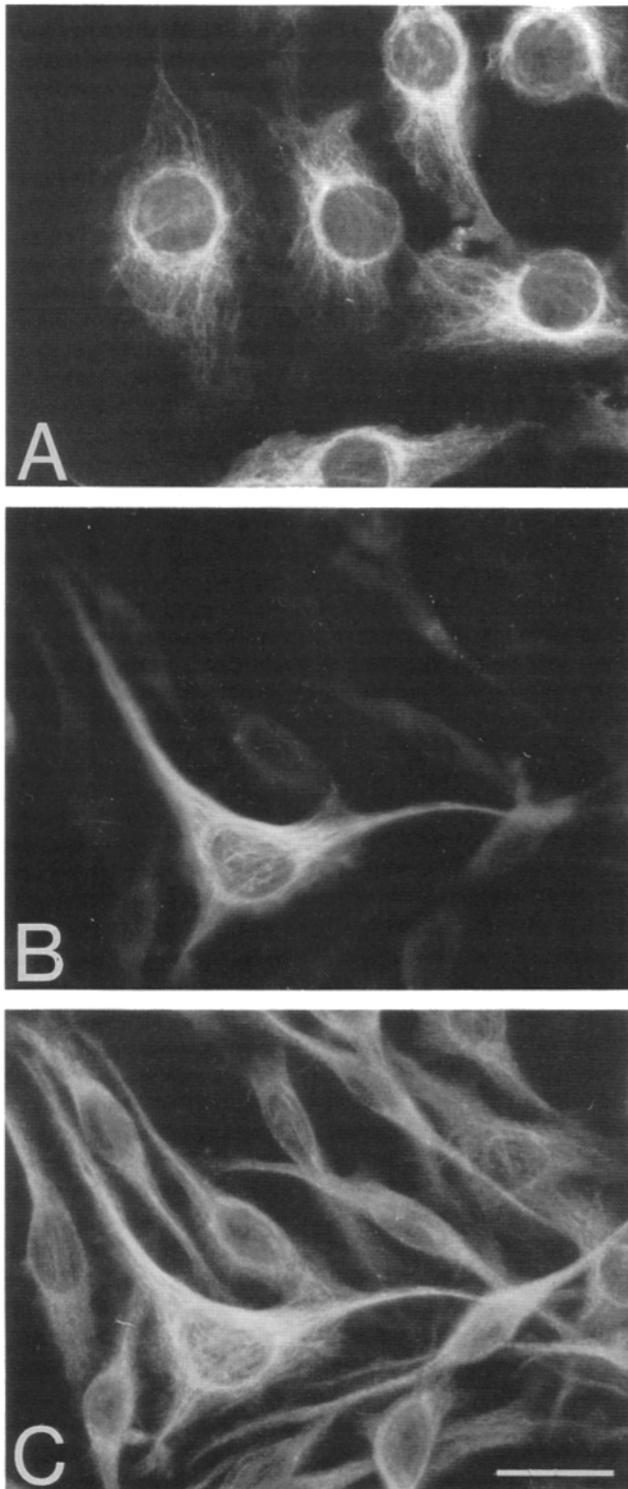
A 2.7-kb GFAP cDNA clone, G92, was isolated from a rat brain  $\lambda$ -gt11 cDNA library by high-stringency DNA hybridization with a fragment of mouse GFAP cDNA (Lewis et al., 1984) and was subsequently subcloned into pGEM-7Zf. The GFAP cDNA insert was further subcloned in both orientations into M13mpl8 for sequencing. Comparison with the published rat GFAP sequence (Feinstein et al., 1992), indicated that the insert was full-length, containing the entire coding and 3'-untranslated regions. Our nucleotide sequence, however, is not completely identical to the published sequence (available from EMBL/Genbank/DBJ under accession number L27219). Nevertheless, most of the differences were located in the 3'-untranslated region. Although two discordant nucleotides were present in the coding region, they did not change the predicted amino acid sequence.

The rat GFAP amino acid sequence showed a high degree of homology with those of human and mouse GFAP. The differences are mainly in the head domain while the tail domains are highly conserved. Even with these high overall homologies, rat GFAP has a lower mobility on SDS-PAGE, by ~2–3 kD, than either human or mouse GFAP (Liem et al., 1978; and Fig. 2).

Further evidence that the G92 cDNA clone is fully encoding for rat GFAP was obtained by *in vitro* transcription/translation after subcloning the GFAP cDNA into pGEM-7Zf. RNA transcripts were generated from either the T7 or SP6 promoter and subsequently translated *in vitro* in a rabbit reticulocyte lysate system. Products of *in vitro* transcription and translation of clone G92 were analyzed on an SDS-PAGE gel, resulting in a band of about 50 kD when the SP6 promoter was used (data not shown).

### Transient and Stable Transfections of L tk<sup>-</sup> Cells Show GFAP-positive Filamentous Networks

Two eukaryotic expression vectors, pRSVi-HindIII and pSV2i-HindIII, were used to express rat GFAP cDNA in cultured cells. Clone G92 GFAP cDNA was isolated from pGEM-G92 and subcloned into the unique HindIII sites in these vectors. The resulting constructs, pRSVi-GFA and pSV2i-GFA, were first tested for eukaryotic cell expression by transient transfections into L tk<sup>-</sup> cells. Stable transfections were performed by co-transfection with pSV2-Neo and selection with the neomycin analog G418. G418-resistant clones were isolated and studied for GFAP expression. All of these stably transfected fibroblasts showed GFAP-positive, filamentous networks reminiscent of those observed in

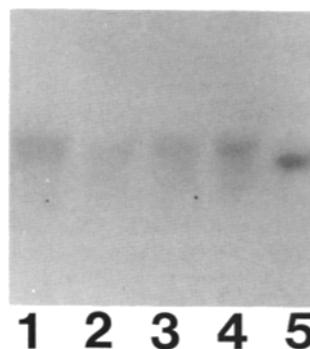


**Figure 1.** Transfected rat GFAP in L tk<sup>-</sup> cells formed filamentous networks reminiscent of those in astrocytic cells. (A) pSV2i-GFA was stably transfected into L tk<sup>-</sup> cells. A representative stable L tk<sup>-</sup> transfectant was stained with polyclonal anti-GFAP antiserum to demonstrate the filamentous networks of rat GFAP. (B and C) The expression of rat GFAP in L tk<sup>-</sup> cells did not change their morphology significantly as demonstrated in the transient transfection experiment which were stained with polyclonal anti-GFAP antiserum (B) and monoclonal anti-vimentin antibody (C). Also notice that GFAP staining co-localizes with the staining of the endogenous vimentin. Bar, 25  $\mu$ M.

astrocytes or astrocytoma cells (Fig. 1 A). The transfected rat GFAP co-localized with the endogenous vimentin network and did not cause significant changes in the morphology of the cells, especially in terms of the quality and quantity of the processes of these fibroblasts which is best demonstrated by the results of transient transfection experiment (Fig. 1, B and C).

#### **Transient and Stable Transfections of AS2-U251 Cells Result in the Reexpression of a Filamentous GFAP Network**

Both pRSVi-GFA and pSV2i-GFA were used to transfect AS2-U251 cells, the GFAP-suppressed cell lines derived from the human astrocytoma cells, U251MG (Weinstein et al., 1991). Transient transfections of either construct into AS2-U251 cells resulted in a GFAP(+) filamentous network. The AS2-U251 lines were obtained by transfection with an expression construct which constitutively transcribed the anti-sense strand of mouse GFAP cDNA and a neomycin resistance selectable marker (Weinstein et al., 1991). The isolation of stable AS2-U251 cell lines reexpressing GFAP therefore required a second selectable marker, for which we used hygromycin-resistance. Plasmids carrying the hygromycin-resistance gene, pUC-Hyg 1.1, were co-transfected with either pRSVi-GFA or pSV2i-GFA in a ratio of 1:20. A number of AS2-U251 transfectants which were resistant to both neomycin and hygromycin and stained positive for GFAP were isolated. Previous studies had shown that there was a difference in gel mobility between human and rat GFAP. To find out whether these cell lines were expressing exogenous rat GFAP or reexpressing human GFAP, we isolated the Triton-insoluble cytoskeletal fraction from four stably transfected clones, ASH3B7E, ASH3D7E, ARH2B8E, and ARH4B9C and studied their GFAP composition by SDS-PAGE and Western blot analysis. The Triton-insoluble cytoskeletal fractions from these clones contained only a single, GFAP(+) band whose mobility was slower than that of human GFAP (Fig. 2). To further substantiate the expression of transfected rat GFAP, total cellular RNA of these stably transfected clones as well as those of U251MG and one of the stably transfected L tk<sup>-</sup> cell lines, GRID6G4, were isolated for RNase protection analyses. As shown in Fig. 3, all four **rescued cell** lines and the stably



**Figure 2.** The GFAP reexpressing stable cell lines contained only rat GFAP. Triton-insoluble fraction of cytoskeletal protein from four GFAP reexpressing stable cell lines, ASH3D7E (lane 1), ASH3B7E (lane 2), ARH2B8E (lane 3), and ARH4B9C (lane 4) were isolated. Equal amounts of pelleted protein were loaded on a 10%, pH 9.2, SDS-PAGE, and transferred overnight to nitrocellulose paper. Monoclonal anti-GFAP antibody and <sup>125</sup>I-labeled goat anti-mouse Ig were used to demonstrate their GFAP content. All four lines showed a single band with a mobility slower than that of human GFAP from the U251MG cells (lane 5).

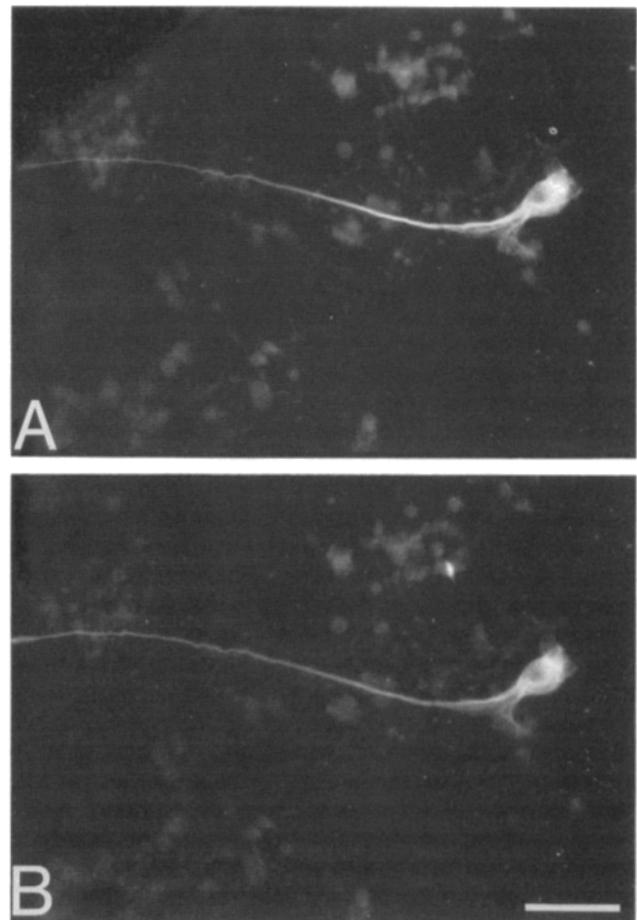
transfected L tk<sup>-</sup> cell line expressed mRNAs which can be fully protected by the antisense riboprobe of rat GFAP. The ARH2B8E cells expressed a second band which may be a truncated rat GFAP mRNA resulting from random integration. In contrast, total cellular RNA from U251MG cells showed only small, discontinuous, protected bands as predicted from the inter-species differences between the two mRNAs. These bands were missing from the four **rescued** cell lines indicating that human GFAP mRNA was not reexpressed in these cells. When RNase protection analyses were done with antisense riboprobe of human GFAP, only the total cellular RNA from U251MG cells contain a fully protected band (not shown). Since neither human GFAP mRNA nor its protein product was detectable in the four **rescued** cell lines, we can rule out the possibility that these clones were revertants.



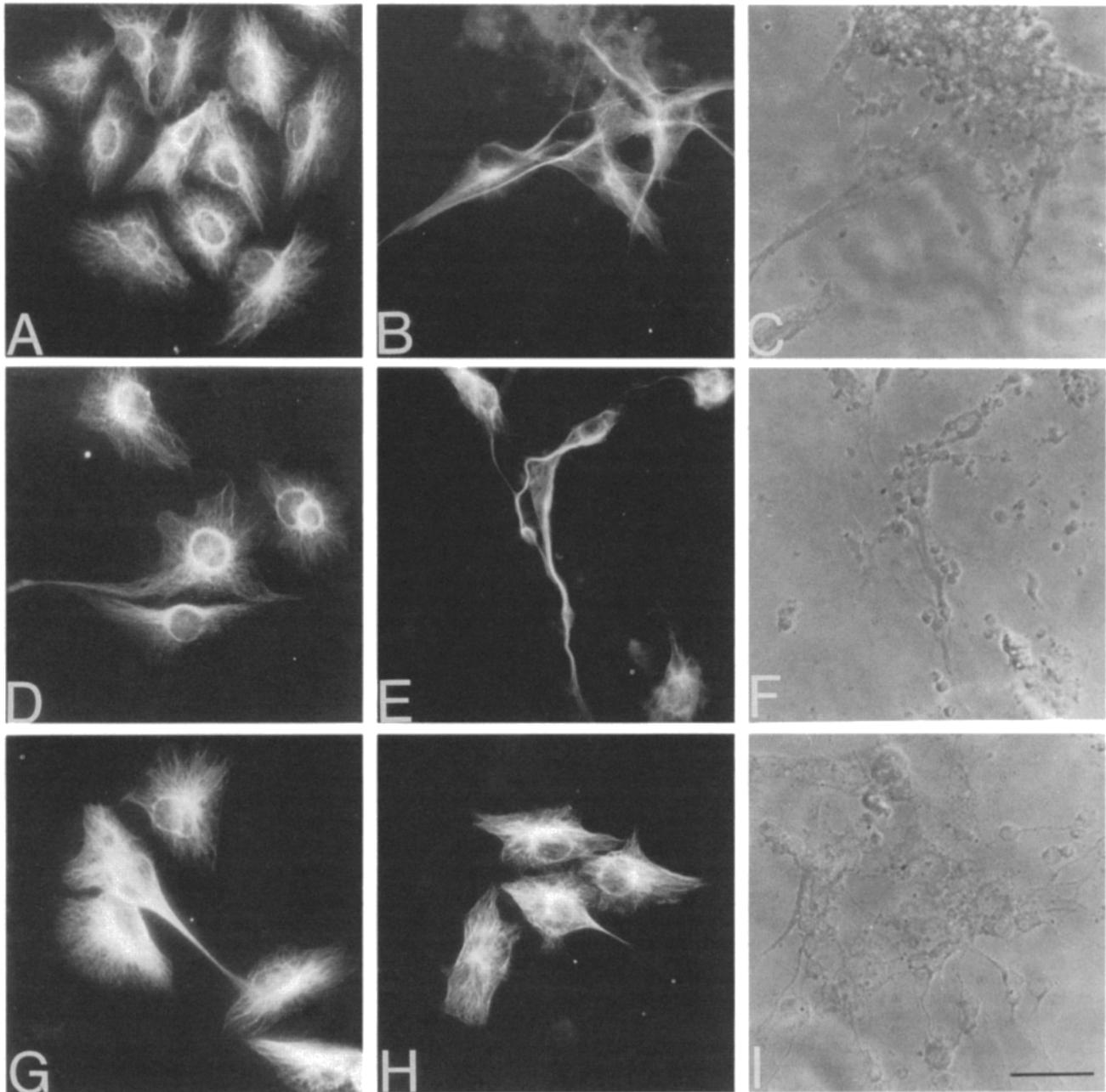
**Figure 3.** RNase protection analyses of total cellular RNA of the **rescued** cell lines shows the presence of rat GFAP mRNA and the absence of human GFAP mRNA. 10  $\mu$ g of total cellular RNA from ASH3B7E (lane 1), ASH3D7E (lane 2), ARH2B8E (lane 3), ARH4B9C (lane 4), U251MG (lane U), GR1D6G4 (lane L) were hybridized with a 1.3-kb antisense riboprobe which contains sequence complementary to the position 1 to 1306 of the rat GFAP mRNA. Lane P is the antisense riboprobe which is transcribed from HindIII-linearized pGEM7Zf-G92(ApaI) using T7 polymerase. Lane M is HaeIII-digested  $\phi$ X174 DNA marker labeled with Klenow DNA polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP. The markers are 1353-, 1078-, 872-, 603-, 310-, and 28-bp long. All the **rescued** cell lines along with the stably transfected L tk<sup>-</sup> cell lines, GR1D6G4, showed a band fully protected by the antisense riboprobe. This band is absent in the human U251MG cells which showed a number of smaller bands not found in the **rescued** cell lines.

### The Reexpression of GFAP in AS2-U251 Cells **Rescues** Their Ability to Form Processes in the Presence of Neurons

Previous studies from our laboratory showed that when GFAP expression is suppressed by means of an anti-sense construct, the resulting mutant U251 cells cannot extend stable processes in response to the addition of neurons. If the suppression of GFAP expression is the sole reason for this loss of function, we should be able to restore it by reexpressing GFAP in these cells. We tested the four cell lines in which GFAP had been reexpressed for their ability to respond to the addition of neurons. Fig. 4 shows an example of one of these **rescued** cell lines co-cultured with primary cerebellar neurons. Also apparent in Fig. 4 is the exact co-localization of GFAP and MAP4 staining (Fig. 4, A and B, respectively), which justified our use of the primate-specific anti-MAP4 antiserum to stain the **rescued** cell lines. As illustrated in Fig. 5, the **rescued** cell lines put out long slender processes comparable to U251MG cells in response to neuronal contact (cf. Fig. 5, B and E), while AS2-U251 cells did not show significant process formation under similar conditions (Fig.



**Figure 4.** The morphological changes of GFAP reexpressing cells in the presence of neurons. The co-culture experiment of one of the **rescued** cell lines, ASH3B7E, with primary granule cells from neonatal rat cerebellum is shown here. The cultures were fixed and stained with polyclonal anti-MAP4 antiserum (A) and monoclonal anti-GFAP antibody, (B), to demonstrate the exact co-distribution of these two proteins. Bar, 50  $\mu$ M.



**Figure 5.** Stable transfectants of AS2-U251 cells showed reappearance of GFAP(+) filamentous networks and **rescue** of process formation in response to neuronal contact. pSV2i-GFA and pRSVi-GFA were stably transfected into GFAP-suppressed AS2-U251 cells. The resulting stable cell lines were co-cultured with primary granule cells from neonatal rat cerebellum. As demonstrated in *E* and *F*, the reexpression of rat GFAP in AS2-U251 cells resulted in longer, stable processes in response to neuronal contact as compared to parental AS2-U251 cells (*H* and *I*). *A*, *D*, and *G* represent, respectively, U251MG, ASH3B7E, one of the **rescued** cell lines, and AHG1A7D, the vector-control cell line, when they were cultured alone. *B*, *E*, and *H* illustrate their morphological change when they were co-cultured with neurons, respectively. *A* through *H*, excluding *C* and *F*, were stained with primate-specific polyclonal anti-antiserum. *C*, *F*, and *I* are phase contrast pictures of *B*, *E*, and *H* to show normal neurite outgrowth of the co-cultured rat cerebellar neurons under all three conditions. Bar, 50  $\mu$ M.

5 *H*). Most of the responding cells had a morphology intermediate between Bergmann-like and stellate forms. However in some local areas, the responding cells displayed relatively complex processes and obtained a stellate morphology (results not shown). The immunofluorescent images were digitized and analyzed with the Image-1 image analysis software. The average process length and number per cell were

measured in the presence and absence of neurons. To avoid counting primary rat brain astrocytes, primate-specific polyclonal anti-MAP4 antiserum was used for immunostaining. As shown in Fig. 4, there was a co-distribution of MAP4 and GFAP and they both reached the very tip of the extending processes. As a consequence, we could stain cells with anti-MAP4 antibody without losing fidelity of their pro-

Table I. Changes of Process Length and Number in the Absence or Presence of Neurons

Cell lines	Relative GFAP content*	Neuron (-)		Neuron (+)		Percentage of increase	
		Average Process Length	Average Process Number	Average Process Length	Average Process Number	Process Length	Process Number
ASH3D7E	120	22.79†	0.67	75.89†	2.04	233	204
ASH3B7E	95	19.60	0.62	74.10	1.91	278	209
ARH2B8E	68	49.77	1.15	129.26	1.78	160	55
ARH4B9C	131	31.38	0.69	130.04	2.24	314	224
AS2	0	33.38	0.80	49.87	0.88	49	9
AHG1A7D	0	41.67	1.04	69.11	1.54	66	48
U251	100	27.97	0.43	89.48	1.62	222	275

The GFAP reexpressing cell lines regained the ability to extend stable processes in the presence of neurons to a degree comparable to U251MG cells. The average process number and length per cell in the presence and absence of neurons were analyzed for four GFAP reexpressing cell lines. The percentage of increase of either process number or process length with the addition of neurons was used to compare between these cell lines and controls to avoid inter-cell line variability. Process measurements performed as described in Materials and Methods.

\* The GFAP content in each cell line is compared to U251 whose GFAP content is arbitrarily designated as 100.

† The unit of process length is  $\mu\text{m}$ .

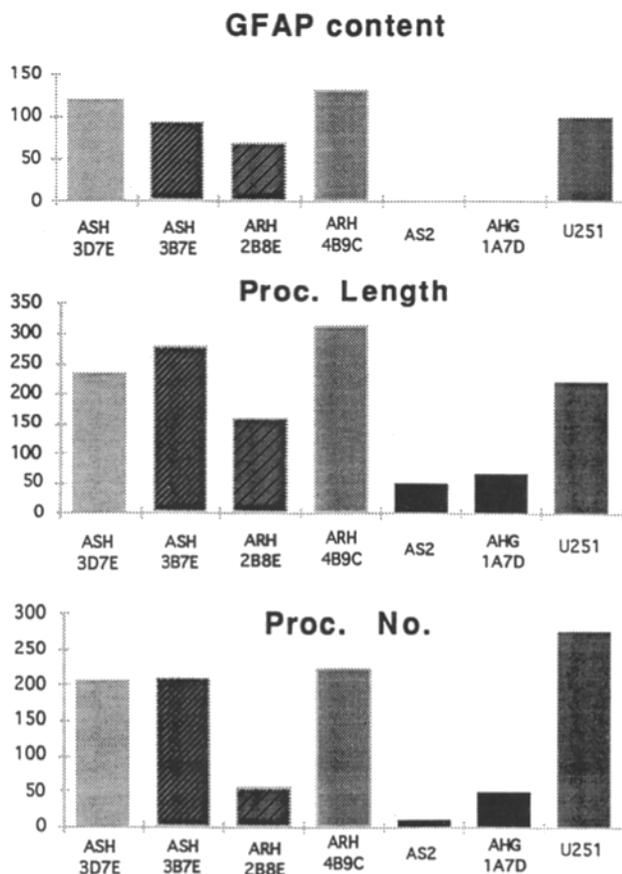


Figure 6. The GFAP reexpressing cell lines put out more and longer processes when co-cultured with neurons. The immunofluorescence image of the four rescued cell lines, ASH3D7E, ASH3B7E, ARH2B8E, and ARH4B9C, two negative-control cell lines, AS2-U251 and AHG1A7D, and a positive-control cell line, U251MG in the presence or absence of granule cells of neonatal rat cerebellum were analyzed as described in Materials and Methods. The average process length and process number per astrocytoma cell in the presence of neurons were compared to those in their absence. The percentages of increase thus obtained (see Table I) were plotted against their relative GFAP content in respect to U251MG cells. All four rescued cell lines increase their process length to a degree comparable to U251MG in the presence of neurons. Excluding ARH2B8E,

processes, both qualitatively and quantitatively. All cell lines examined, including AS2-U251 and the vector-control cell line AHG1A7D, showed increases in process length when co-cultured with neurons. To avoid the inherent variations in process formation capabilities among the transfected cell lines, we compared percentages of increase upon addition of neurons rather than the raw numbers. The percentages of process length increase obtained for the rescued cell lines varied from 160 to 314% (Table I). In comparison, the percentages of increase in process length of AS2-U251 and AHG1A7D cells were 49 and 66%, respectively. Therefore, all four GFAP reexpressing cell lines extended longer processes in response to neurons than either AS2-U251 or control AHG1A7D cells. The percentage of process length increase displayed by the GFAP reexpressing lines was also comparable to that of the parental, untransfected U251MG cells (220%). A correlation between the amount of GFAP expressed and the length of the processes was also observed (Fig. 6). The average process number per cell of these GFAP reexpressing lines also increased in the presence of neurons (Table I, and Fig. 6). However, ARH2B8E did not show an increase in process number comparable to the other three GFAP reexpressing cell lines. Collectively, we conclude that three of the four lines tested completely regained the ability to extend processes in response to neurons.

## Discussion

Although intermediate filament proteins show a cell type-specific distribution, no cell type-specific functions have as yet been directly demonstrated for most of these proteins. Several lines of evidence indicate that GFAP should have such a specific function. Firstly, astrocytes in different regions of the adult central nervous system contain different

their average process number per cell also increased to a degree comparable to U251MG. There seemed to be a correlation between the amount of GFAP and the ability to extend longer processes but the process number increase did not correlate well with GFAP amount.

levels of GFAP (Gasser and Hatten, 1990b; Kretschmar et al., 1985). Protoplasmic astrocytes in gray matter, which normally contain little GFAP, accumulate the protein rapidly in response to a variety of insults (Bignami and Dahl, 1976; Amaducci et al., 1981; Goldmuntz et al., 1986; Smith and Eng, 1987; Smith et al., 1983). Secondly, GFAP has been found to accumulate during postnatal brain development in parallel with the transition from a motile to a non-motile cell in culture (Fedoroff, 1986; Kalnins et al., 1984). Thirdly, a transient elevation in both GFAP mRNA and immunoreactivity which corresponds to the proliferation and differentiation of interfascicular glia occurs after cessation of axon ingrowth (Schnitzer et al., 1981) and during initiation of myelination in the white matter of postnatal mouse cerebellum (Bovolenta et al., 1984). Fourthly, Bergmann fibers in the developing mouse cerebellum are stained positive for vimentin at E19 and become GFAP immunoreactive after the first postnatal week when granule cells of the external granular layer start to migrate inward (Bovolenta et al., 1984). Similar findings that GFAP(+) cells appeared at the time of glial-guided migration were reported in the hippocampus and other regions of the developing rodent brain (Gasser and Hatten, 1990a; Mason and Gregory, 1984).

Finally, the suppression of GFAP expression in human U251MG astrocytoma cells caused the loss of the ability of these cells to form stable complex processes in the presence of granule neurons (Weinstein et al., 1991). Since the expression of vimentin and other cytoskeletal elements in these AS2-U251 cells were not affected, this result was the first direct evidence that GFAP has a unique role in the process extension of these astrocytic cells.

To establish with greater certainty that the inability of the GFAP-suppressed cells was solely due to the absence of GFAP, we set out to **rescue** the ability of these cells to extend stable processes by expressing rat GFAP in these cell lines. We used rat GFAP instead of human GFAP in order to rule out the possibility of wild type revertants, which would express human GFAP. Toward this end, we screened a rat brain cDNA library and obtained a fully encoding rat GFAP cDNA which included the entire 3'-untranslated region. This clone was transfected into the GFAP-suppressed AS2-U251MG cell lines previously created in our laboratory. The results showed that not only did a GFAP-containing filamentous network re-appear, but process formation in response to neurons was restored.

Four stable transfectants were randomly selected and tested for their process extending ability in the presence of granule neurons. The degree of response varied among these isolates. All four responded as well or better than U251MG cells when average increases in process length were compared. However, one isolate, ARH2B8E, failed to show a significant response in terms of increases in process number to neuronal stimulation. Taking into account the fact that these **rescued** cell lines had gone through two stable transfection events and were over-expressing three foreign proteins, rat GFAP, aminoglycoside phosphotransferase, and hygromycin B phosphotransferase, it is possible that one of the stable transfectant lines did not fully regain the ability to extend processes in response to the contact of neurons. We believe that these results further substantiate the role of GFAP in true process extension of these astrocytoma cells when they are co-cultured with granule neurons of rodent cerebellum.

As shown in Fig. 6 and Table I, these **rescued** cell lines also expressed different amounts of GFAP. Although there was no linear correlation between the amount of GFAP expressed and the degree of process extension, the high amount of GFAP in one cell line, ARH4B9C, seemed to be correlated to its better ability to extend processes in the presence of neurons and, in contrast, the poorest responding cell line, ARH2B8E, expressed the lowest amount of GFAP.

The AS2-U251 cells were created by transfecting the antisense-strand of the protein coding region of the mouse GFAP into the U251MG cells. To overcome the effect of antisense mRNA and reexpress GFAP in these AS2-U251 cells, we over-expressed the sense strand of rat GFAP by the same strong, constitutive promoter that was used to drive the expression of antisense mRNA, which may be one of the reasons why our **rescued** cell lines showed only rat GFAP mRNA without detectable amounts of the endogenous human GFAP mRNA.

We were also concerned about the non-specific effects of any exogenous oligonucleotide. As has been pointed out by Woolf et al. (1992), the shortest sequence that is likely to be unique within the mRNA pool is 13 bases. In other words, any sequence less than 13 bases in length is expected to occur more than once in the mRNA pool. Any oligonucleotide longer than 13 bases will contain more than one consecutive 12 mer and hybridize with more species of mRNA. Therefore, any antisense oligonucleotide designed for a particular mRNA is expected to have non-specific effects on other unrelated mRNAs. By this argument, a constitutively expressed antisense mRNA such as the one we used in AS2-U251 cells which contains more than one thousand consecutive 12 mers could result in a thousand times more non-specific effects. However, it is clear that the longer the sequence of the antisense mRNA, the less chance there is to find an unrelated mRNA with more than 10% fully complementary sequence in the mRNA pool. As a consequence, the melting temperature between these two species of mRNA may be so low that any significant hybrid formation between the two mRNAs will not exist at the physiological temperature of the cell. In addition, living cells renew their mRNA constantly. Compared to the specific effect of antisense mRNA, the insignificant non-specific effect on other unrelated mRNAs will be further minimized by the cellular renewal of these mRNAs. Nevertheless, the non-specific effect of antisense mRNA cannot be ruled out completely. Therefore, be it unlikely, there remains a possibility that the mRNA pool of AS2-U251 may have been disturbed by the non-specific effects of the antisense mouse GFAP mRNA and the transfection of sense strand rat GFAP mRNA may have titrated out these non-specific suppressions and resulted in the **rescue** of the phenotype. This possibility is deemed even more unlikely since the transfection of the rat GFAP failed to unblock the specific suppression of human GFAP mRNA.

Two different forms of glia have been described in early postnatal mouse cerebellum: stellate and elongated, Bergmann-like glia (Hatten et al., 1984). These two cell types interact differently with neurons. The stellate forms usually associate with several dozen neuronal cells while the elongated, Bergmann-like glia react with two to three neurons. In vitro studies with either hippocampal or cerebellar neu-

rons showed that most migration occurs on bipolar or elongated Bergmann-like glial profiles (Gasser and Hatten, 1990a; Hatten et al., 1984). Although in the co-culture experiments of primary astrocytes and granule neurons, most astrocytes had a stellate profile (Hatten et al., 1984), pure stellate forms are uncommon in our **rescued** cell lines. Most of our responding cells are intermediate between Bergmann-like and stellate cells, with one prominent, elongated process accompanied by one or two shorter processes. These are closer in morphology to the bipolar form of astroglia in cultures derived from the developing rat hippocampus (Gasser and Hatten, 1990a). According to the study of Misson et al. (1991) on murine cerebral cortex, this form of astroglia is ontogenically earlier than the other forms. This may be related to the de-differentiated state of these astrocytoma-derived cell lines.

Process formation involves many phases of interaction but can roughly be separated into three phases: initiation, elongation, and consolidation/stabilization (Alleta and Greene, 1988; Goldberg and Burmeister, 1992; Teng and Greene, 1993). Actin networks and their associated proteins appeared to be involved in initiation (Fedoroff et al., 1987; Goldman and Abramson, 1990) while the extracellular matrix and membranous proteins play a major part in leading, guiding and ending a process in extension phase (Mason et al., 1988; Streit et al., 1993; Stitt and Hatten, 1990; Antonicek et al., 1987). Besides contact with live neurons (Hatten, 1985; Hatten, 1987; Weinstein et al., 1990, 1991), cAMP analogues such as dibutyryl cAMP (Goldman and Abramson, 1990; Goldman and Chiu, 1984; Messens and Slegers, 1992; Fedoroff et al., 1987), brain extract (Pollenz and McCarthy, 1986; Lim et al., 1973), phorbol ester (Harrison and Mobley, 1990), cytochalasin B (Baorta et al., 1992), prostaglandins (Tardy et al., 1981), lipopolysaccharide (Messens and Slegers, 1992), and hormonal stimulus (Choi and Lapham, 1978) have all been reported to induce astrocytes or astrocytoma cell process formation. Among them, agents or factors expected to increase intracellular cAMP such as dibutyryl cAMP, forskolin (Pollenz and McCarthy, 1986; Shafit-Zagardo et al., 1988), and  $\beta$ -receptor agonists (Browning and Ruina, 1984; McCarthy et al., 1985; Murphy and Pearce, 1987; Shain et al., 1987) have more consistent effects on cell morphology. GFAP protein (Backhovens et al., 1987; Goldman and Chiu, 1984; Messens and Slegers, 1992) and mRNA (Shafit-Zagardo et al., 1988; Messens and Slegers, 1992) in these astrocytes or astrocytoma cells are usually elevated in response to increased intracellular cAMP and increased phosphorylation of GFAP and vimentin is a consistent finding preceding either cAMP- or phorbol ester-induced changes in astrocyte morphology (Pollenz and McCarthy, 1986; Baorta et al., 1992; Harrison and Mobley, 1990), indicating that there could be a role for IF's in the formation of these processes. However, it is unlikely that changes of IF's, including their synthesis and posttranslational modification such as phosphorylation, are involved in process initiation because astrocyte process formation can be dissociated from these changes of IFs (Pollenz and McCarthy, 1986; Baorta et al., 1992).

Our data argue that GFAP is an indispensable part of the formation of stable processes of U251 astrocytoma cells in response to neuronal contact, since the suppression of GFAP led to a decrease in process formation and its reexpression

**rescued** this phenomenon. However, both the GFAP-suppressed AS2-U251 cells and the vector-only control cell line, AHGIA7D, showed some degree of process formation in response to neuronal contact (Table I and Fig. 6). Given that IFs may not be required for initiation, one explanation could be that the other cytoskeletal elements, such as microtubules and microfilaments, are capable of initiating and extending processes in response to the neuronal stimuli but cannot stabilize these processes in the absence of GFAP. Most likely, the processes are constantly extending and retracting and GFAP is required to stabilize the processes, rendering them more resistant to retractions (Fedoroff et al., 1987; Duffy, 1983). Although these results argue for a role of GFAP in the stabilization of astrocytic processes in cultured cells, we have to be careful in extending this conclusion to a real *in vivo* situation, since similar conclusions were made for the role of the microtubule associated protein, tau, in the formation of axonal processes in primary neurons in culture (Caceres and Kosik, 1990). Cerebellar neurons in dissociated culture become polarized and extend characteristic single, axonlike neurites after a relatively constant interval. By selectively inhibiting tau expression with antisense oligonucleotides, the elaboration of axonlike neurites was significantly suppressed. Since the initial exploratory neurite formation was not affected, tau seemed to play an indispensable role in the conversion (stabilization) phase of axonal development. In contrast, gene targeting studies showed that the nervous system of tau-deficient mice appeared to be normal immunohistologically and axonal elongation was not affected in cultures of primary neurons from these mice (Harada et al., 1994). These results clearly indicate that tau is dispensable for axonal elongation *in vivo*. Similar contradictions between the results of antisense suppression studies and those of transgenic knockout studies have also been reported in another system (Sariola et al., 1991; Lee et al., 1992). Our conclusions about the role of GFAP in astrocytic process formation *in vivo* must therefore await the results of specific gene targeting studies in transgenic mice.

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