Suppression by Antisense mRNA Demonstrates a Requirement for the Glial Fibrillary Acidic Protein in the Formation of Stable Astrocytic Processes in Response to Neurons

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Abstract. The glial fibrillary acidic protein (GFAP) is a glial-specific intermediate filament protein, which is expressed in astrocytes in the central nervous system, as well as in astrocytoma cell lines. To investigate the function of GFAP, we have studied the human astrocytoma cell line, U251, which constitutively expresses GFAP and vimentin in the same 10-nm filaments. These cells respond to neurons in vitro in the same way as primary astrocytes: they withdraw from the cell cycle, support neuronal cell survival and neurite outgrowth, and they extend complex, GFAP-positive processes. To determine the role of GFAP in these responses, we have specifically suppressed its expression by stably transfecting the U251 cells with an antisense GFAP construct. Two stable antisense cell lines from separate transfections were isolated and were shown to be GFAP negative by Northern and Western blot analyses, and by immunofluorescence studies. The antisense cell lines were inhibited in their ability to extend significant glial processes in response to neurons. In culture with primary neurons, the average increase in process length of the U251 cells was nearly 400%, as compared to only 14% for the antisense transfectants. The other neuron induced responses of astrocytes, i.e., proliferative arrest and neuronal support, were not affected in these cell lines. These data support the conclusion that the glial-specific intermediate filament protein, GFAP, is required for the formation of stable astrocytic processes in response to neurons.

INTERMEDIATE filaments are 8–10 nm cytoskeletal structures composed of a class of proteins exhibiting a high degree of cell-type specificity. Thus neurofilaments are found in neuronal cells, desmin in muscle cells, the keratins in epithelial cells, whereas astrocytes express the glial specific, glial fibrillary acidic protein (GFAP).[1] In addition, vimentin, which is present in cells of mesenchymal origin can be expressed in astrocytes, as well as in most cells in tissue culture. The functions of the intermediate filament proteins are unknown, although their tissue specificity suggests that they are involved in specialized functions related to the differentiation state of the cell.

During development of the central nervous system, neurons are born at the ventricular surface and then migrate along astrocytic processes toward their ultimate destination, where astrocytes provide the scaffolding and trophic support required for neuronal positioning (Rakic, 1972, 1981; Hatten et al., 1988). These observations suggest that the extension of astrocytic processes, which are rich in GFAP, is critical to CNS organogenesis.

In tissue culture systems, primary astrocytes respond to the presence of neurons by withdrawing from the cell cycle, extending complex processes, and promoting neuronal survival and outgrowth (Hatten and Shelanski, 1988; Hatten, 1987; Gasser and Hatten, 1990). These responses appear to resemble the behavior of primary astrocytes in situ. Most prominent among these neuron-induced responses is the extension of lengthy and complex GFAP-positive processes; astrocytic cells grown in the absence of neurons have a flat, fibroblastic appearance, with little or no process formation (Hatten, 1985). A number of rodent and human astrocytoma cell lines display similar neuron-induced responses in vitro (Hatten and Shelanski, 1988). One of these, the human astrocytoma cell line U251, constitutively expresses GFAP and vimentin in the same 10-nm filaments (Westermark, 1973; Wang et al., 1984).

To investigate the role of GFAP in these phenomena, we specifically interfered with GFAP expression in U251 cells by stably transfecting them with an antisense construct of the GFAP gene and characterized the responses of the GFAP-negative (AS) cells to the addition of neurons. The data show that the AS cells are unable to express GFAP, even in the presence of neurons, and that unlike the parent U251 cell line, they do not establish the complex, stable processes in response to neurons. The absence of GFAP and astrocytic processes, were the only observed alterations in these stable transfectants.

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1. *Abbreviations used in this paper:* AS, glial fibrillary acid protein-negative; GFAP, glial fibrillary acidic protein; PD, phosphate-buffered saline without calcium and magnesium; MAP, anti-microtubule-associated protein.

Materials and Methods

Plasmid Construction and Transfection

All molecular biology buffers, reagents and enzymes were purchased from Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN. The coding region of the murine GFAP cDNA (Lewis et al., 1984) was excised from the pUC9 vector at the Sal I–Eco RI restriction sites, and the 1.2-kb fragment was purified as described (Vogelstein and Gillespie, 1979). The ends were filled in with the large Klenow fragment of *Escherichia coli* DNA polymerase I, and Hind III linkers were added, trimmed back, and annealed to the pSV2i plasmid (Chin and Liem, 1989), which had been cut at a unique Hind III restriction site (the plasmid was the generous gift of Drs. H. Samuels and B. Forman, New York University Medical Center, New York). Competent HB101 cells were transformed with this plasmid, and plated on ampicillin plates. Transformants were picked and transferred to nitrocellulose filters, which were then hybridized with a ^{32}P nick-translated GFAP coding cDNA (Davis et al., 1986). Colonies that hybridized with the probe were expanded and plasmid analysis was carried out (Taylor and Brose, 1988). The orientation of the pSV2i/GFAP plasmid in the transformants was determined by restriction enzyme analysis. The antisense orientation plasmid, pSV2i/as-GFAP, was expanded and purified. The plasmid was linearized at a unique Bam HI site, and the neo resistance gene under the control of the SV-40 promoter was inserted as a cartridge (Chin and Liem, 1989), resulting in the new plasmid, pSV2neo/asGFAP. Bacterial cells were infected with this plasmid, and the cycle of analysis, purification, and expansion was repeated. The plasmid was linearized at the unique Eco RI site and the linear DNA was transfected into U251 cells using the calcium phosphate method (Wigler et al., 1979).

3 d after transfection, the cells were switched into selection medium that contained 1 mg/ml of the neomycin analogue G418 (Gibco Laboratories, Grand Island, NY). Foci of G418 resistant cells were picked using a cloning ring, and expanded. Colonies were evaluated for the expression of the GFAP gene product by immunofluorescence. Colonies that were virtually nonreactive with the anti-GFAP antibody were cloned to the single cell stage (Mellman et al., 1980), and reevaluated by Northern blot, Western blot, and immunofluorescence analyses. In all cases, U251 cells transfected only with the neomycin resistance gene were included as controls.

Northern Blot Analysis

Total RNA was isolated from each cell line on CsCl gradients, and 20 mg of material were electrophoretically separated and transferred to a Genescreen nylon filter (New England Nuclear, Boston, MA), as previously described (Davis et al., 1986). The filters were probed with ^{32}P nick-translated cDNA of either the 5′ end of the mouse GFAP gene (Lewis et al., 1984), or the 5′ end of the human vimentin gene (the kind gift of Dr. R. Baserga, Temple University), overnight at 65°C. The filters were then washed sequentially at 65°C, three times in 2× SSC, 0.1% SDS; twice in 1× SSC, 0.1% SDS; and once in 0.1× SSC, 0.1% SDS, dried, and exposed to x-ray film.

Western Blot Analysis

Cytoskeletal preparation of U251 and AS cells were generated essentially as described (Chin and Liem, 1989). Briefly, cells were grown to confluence, washed extensively with PBS, scraped from the flask, pelleted, and washed twice with PBS. The cells were extracted with 1% Triton X-100 in phosphate buffered saline without calcium and magnesium (PD), with 2 mM phenylmethylsulfonylfluoride; the extracts were treated for 30 min at room temperature with 0.5 mg/ml of DNAase in PD with 10 mM CaCl$_2$ (all from Sigma Chemical Co., St. Louis, MO). The Triton-insoluble cytoskeletal extracts were pelleted and resuspended in SDS-PAGE sample buffer and equal loads of sample were loaded onto a 7.5% polyacrylamide gel and run at constant current (Laemmli, 1970). The proteins were electroblotted onto nitrocellulose paper in 10 mM Caps buffer, 10% MeOH, pH 11.4 (Matsudaira, 1987). The blots were probed with monoclonal anti-GFAP and anti-vimentin antibodies followed by peroxidase-conjugated goat anti-mouse second antibody (Boehringer Mannheim Biochemicals, Inc.). The reaction product was visualized with diaminobenzidine (Sigma Chemical Co.) and hydrogen peroxide.

Cell Culture

Astrocytoma cells were grown in T75 tissue culture flasks (Falcon Labware,

Becton, Dickinson & Co., Lincoln Park, NJ) in Dulbecco's minimal essential medium, supplemented with 10% heat-inactivated fetal calf serum, 5% heat-inactivated horse serum, MEM nonessential amino acids, penicillin, and streptomycin (all from Gibco Laboratories) and 20 mM Hepes (Sigma Chemical Co.). The AS cell lines were grown in the above medium supplemented with 1 mg/ml of G418 (Gibco Laboratories). All cultures were maintained at 37°C and 7% CO$_2$ at controlled humidity.

Primary cerebellar granule cell neurons were prepared from postnatal day-4 Sprague Dawley rats, as described by Hatten (1987). The purity of the preparations was assessed by staining with anti-GFAP antibodies. Astrocyte contamination in the neuronal preparations was always 1% or less.

The C17 cells are a retrovirally transformed preneuronal cell line which has been described elsewhere (Weinstein et al., 1990; Ryder et al., 1990).

Unless otherwise specified, all tissue culture assays were carried out in Lab-Tek 8-well tissue culture slides (Miles Laboratories Inc., Naperville, IL), in 0.2–0.4 ml of complete medium. The slides were coated with 100 mg/ml of poly-D-lysine (Sigma Chemical Co.). Cells were incubated at 37°C in 7% CO$_2$ at controlled humidity.

In coculture experiments, the astrocytic cells were mixed with culture partners in suspension, and the cells were allowed to settle to the substrate.

Immunocytochemistry

Cells were grown described above for 24–48 h, after which the cultures were fixed. Cells to be evaluated for the presence of internal antigens were fixed in ice cold methanol at −20°C for 7 min and washed by immersion in PD for 20 min. Nonspecific binding was blocked by incubation in a solution of 10% fetal calf serum and 10% normal goat serum in PD for 30 min at room temperature. The blocking solution was removed and primary antibodies were added for 60 min at room temperature. The slides were washed by immersion in PD for 1 h, then incubated for 20 min at room temperature in fluorescein- or rhodamine-conjugated goat anti-rabbit or goat anti-mouse Ig (Antibodies, Inc., Davis, CA), diluted to a final concentration of 1:300, and washed a final time in PD for 1 h. The slides were mounted in 16% polyvinyl alcohol (Sigma Chemical Co.), 33% glycerol in PD, and viewed under a Nikon Optiphot microscope fitted for fluorescence microscopy. In double-labeling experiments the slides were treated identically except that both primary antibodies were added simultaneously, followed by a cocktail of both secondary antibodies.

Rabbit polyclonal anti-glial filament antibody that specifically recognizes the GFAP molecule was used at a final concentration of 1:200 (Wang et al., 1984). In double-labeling experiments, we visualized the GFAP positive cells with a specific rabbit antiserum diluted 1:100, or with a mouse monoclonal antibody, G-A-5 (Boehringer Mannheim Diagnostics, Inc., Houston, TX), diluted 1:50. To image vimentin in the cells we used an anti-vimentin rabbit antiserum diluted 1:100 (Wang et al., 1984), or a monoclonal mouse antibody specific for the vimentin molecule at a final concentration of 1:50 (Boehringer Mannheim Diagnostics, Inc.). Anti-microtubule–associated protein 4 (MAP4) is a rabbit antiserum that specifically recognizes the primate 210-kD MAP. It was raised as described by Bulinski and Borisy (1980) and was the kind gift of Dr. S. Chapin (Columbia University, New York). Neurites were stained with NF2B3, a monoclonal antibody that reacts with the high molecular mass neurofilament subunit (Liem et al., 1985).

Evaluation of Glial Proliferation

In [^{3}H]thymidine incorporation assays, U251 cells were plated 8 × 10^4 to 1 × 10^5 cells/well in 1 ml of complete medium in 24-well tissue culture plates (Falcon Labware), followed immediately by membrane preparations of C17 cells at a final concentration of 10 mg/ml (Weinstein et al., 1990). 16 h before harvesting, 1.0 mCi of tritiated thymidine was added to each well. Cells were harvested by trypsinization, and immediately upon release from the substrate four times the volume of complete medium was added and the cells pelleted. Each cell pellet was resuspended in 1 ml of trichloroacetic acid and filtered onto disk filter (GF/C; Whatman, Mainstone, UK). Each experimental point was done in triplicate. The standard error of the mean was always <15%. The radioactive incorporation in U251 cells cultured in the absence of effector cell membranes was considered as 100% incorporation (control). Percent incorporation was calculated as 100 × (mean of the counts in the experimental group/control).

Evaluation of Astrocytoma Process Outgrowth

U251 cells or AS cells were cultured with purified primary granule cell neurons from cerebellum. After 48 h the cultures were fixed and double stained

with the anti-MAP4 antibody and anti-GFAP monoclonal antibody. Double immunostaining was used to insure that the AS cells did not express GFAP in the presence of neurons, and to assess the purity of the granule cell neuron preparation. Random fields ($n > 300$) were imaged by immunofluorescence microscopy and the images were recorded on a video optical disk recorder as described (Dunn et al., 1989). The optical disk image was later replayed with the observer blinded to culture conditions. The length of the processes of the MAP4 positive cells was taken as the distance from the tip of a process to the middle of the cell soma. In cells that had more than one process, each was evaluated. All the MAP4 positive cells in the field were evaluated. Double staining of each field was evaluated by switching from rhodamine to fluorescein excitation wavelengths.

Results

U251 Cells Stably Transfected with an Atisense Construct of the GFAP Gene Fail to React with Anti-GFAP Antibodies

U251 cells constitutively express the astrocyte specific intermediate filament protein, GFAP. In an effort to interfere with the expression of this protein we stably expressed antisense mRNA of the GFAP gene in the U251 cells. The pSV2i/as-GFAP construct (Fig. 1) was introduced into the parent cell line by the calcium phosphate precipitation method (Lewis et al., 1984; Wigler et al., 1979). Transcription of both the antisense GFAP and the neomycin selection marker were driven by the constitutive SV-40 large T promoter (Chin and Liem, 1989). Selection with the neomycin analogue G418 was maintained continuously, beginning 3 d subsequent to transfection. Antibiotic-resistant colonies were randomly picked, expanded, and screened for the expression of GFAP by immunofluorescence. Colonies that were negative by the initial screen were cloned to the single cell stage, expanded, and rescreened for the loss of GFAP expression. These colonies were termed the AS cell lines. Cells transfection with an identical plasmid that contained only the neo resistance marker and its viral promoter behaved in all respects in the same manner as the untransfected U251 cells. There was no observable effect on intermediate filament expression, cell proliferation when neurons were added, the ability of the cells to support neurons, or to form long, stable processes when neurons were added. Two GFAP-negative cell lines, AS2 and AS3, each from separate transfection experiments, were characterized in detail.

AS2 and AS3 failed to react with a polyclonal rabbit antibody that specifically recognizes the GFAP protein (Fig. 2, c and e), whereas U251 cells stain intensely with the antiserum (Fig. 2 a). All the lines continue to react with a monoclonal antibody directed against vimentin (Fig. 2, b, d, and f).

Western blot analysis of cytoskeletal preparation of U251 cells using G-A-5 anti-GFAP monoclonal antibody revealed a major band that migrated with an apparent molecular mass of 55,000 D, while AS2 and AS3 cytoskeletal preparations failed to react with the antibody (Fig. 3). The same preparations from all three cell lines appeared to react equally with a monoclonal antibody against vimentin (Fig. 3, lanes 4–6).

Expression of Antisense GFAP mRNA in Astrocytoma Cells Results in the Loss of Hybridization to the Native GFAP mRNA

Northern blot analysis confirmed the immunofluorescence data. Overnight exposure of a blot probed with a nick-trans-
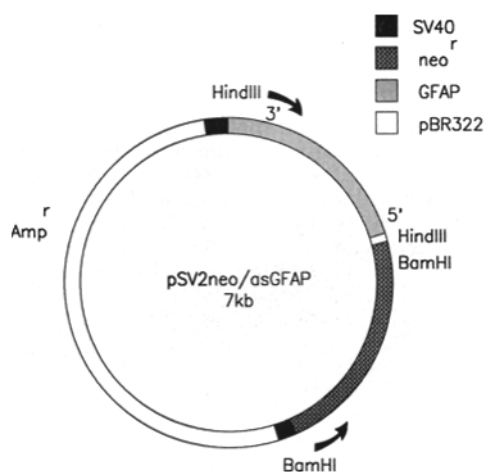

Figure 1. The plasmid used to introduce the antisense GFAP gene into U251 cells. Transcription of the asGFAP and the neomycin resistance selectable marker are driven by separate viral SV-40 early promoter sequences. The remaining plasmid DNA sequences encode the bacterial ampicillin resistance gene and stop codons. The arrows indicate the direction of transcription.

lated GFAP cDNA with mRNA showed hybridization from the parent cells, but not with mRNA from the AS2 or AS3 cells (Fig. 4, lanes *1–3*). Fig 4, lanes *4–6* show the same filter exposed for 3 d, revealing no further hybridization of the mRNA from the AS2 or AS3 cells with the GFAP probe. mRNA from the parent cell line, as well as the asGFAP cells, hybridized strongly to a cDNA probe for vimentin (Fig. 4, lanes *7–9*, overnight exposure). The absence of any signal for GFAP in the AS2 and AS3 cells could be due to the degradation of double-stranded RNA.

AS Cells Are Contact Inhibited by Neurons

Neurons and neuronal membrane preparations have been shown to cause primary astrocytes and many astrocytoma cells, including the U251 cell line, to exit the cell cycle by a contact mediated phenomenon (Hatten and Shelanski, 1988; Hatten, 1987; Weinstein et al., 1990). This effect appears to be restricted to neurons of CNS origin (Weinstein et al., 1990). To test whether the expression of the antisense mRNA and the subsequent loss of GFAP gene product had an effect on this aspect of neuron–astrocyte interaction, we mixed AS cells or U251 cells with neuronal membranes. Proliferation was measured as a function of tritiated thymidine incorporation. As shown in Table I, the AS cells and the U251 cells rapidly become quiescent in response to neuronal membranes. In the absence of neuronal membranes, the cells continued to incorporate label.

AS Cells and U251 Cells Are Indistinguishable in their Ability to Promote Neuronal Survival and Neurite Outgrowth

Neuronal survival and neurite extension is dependent on the presence of astrocytes or soluble factors synthesized by these cells. AS2 and AS3, as well as the control astrocytic cells supported survival and neurite outgrowth of neonatal granule cell neurons from cerebellum. We established cocultures of granule cell neurons and U251 astrocytoma cells, AS2 or
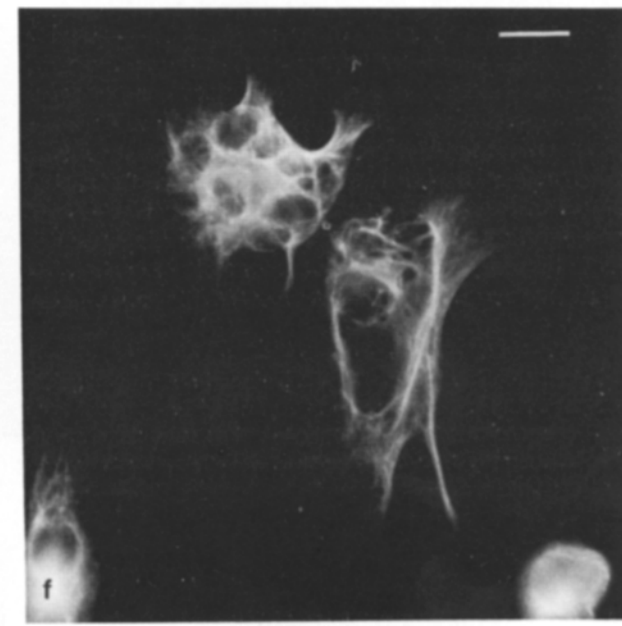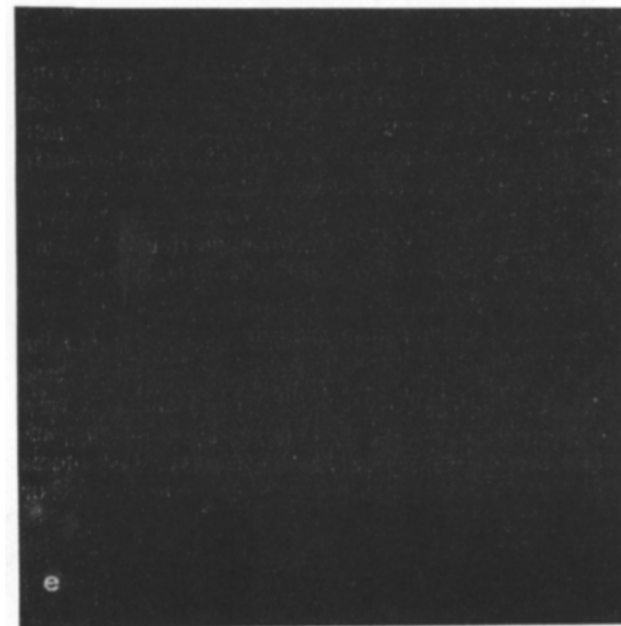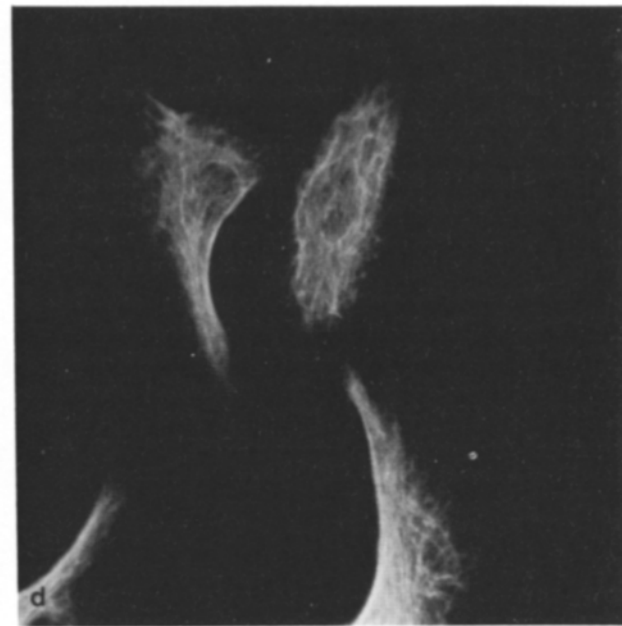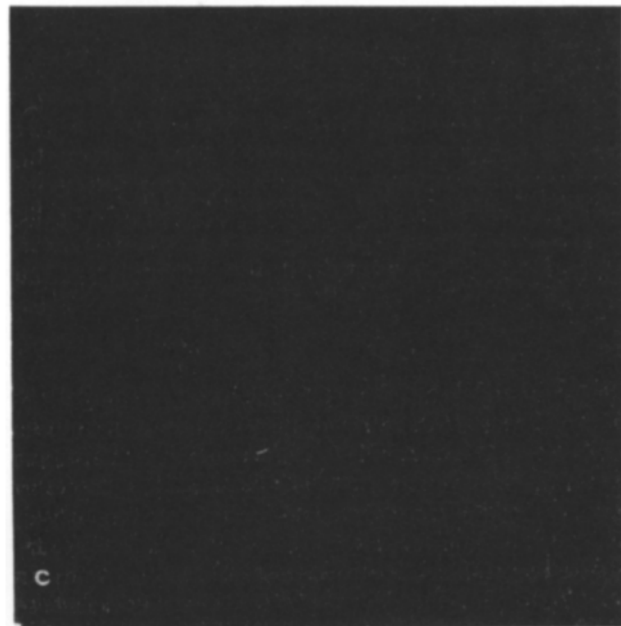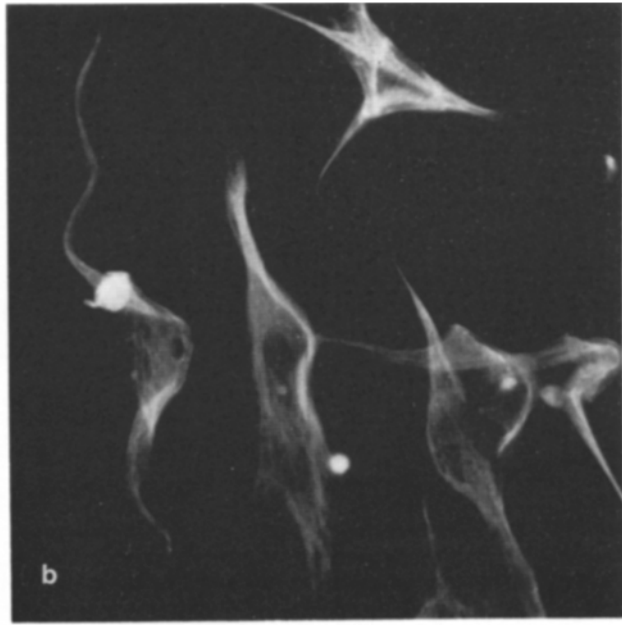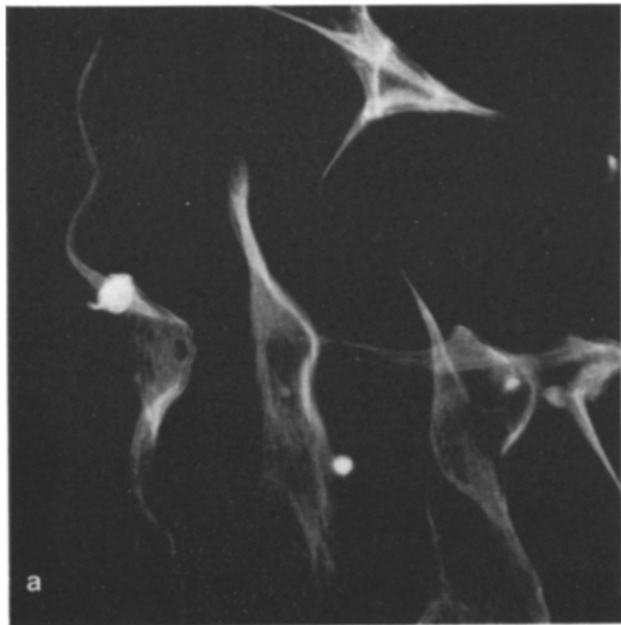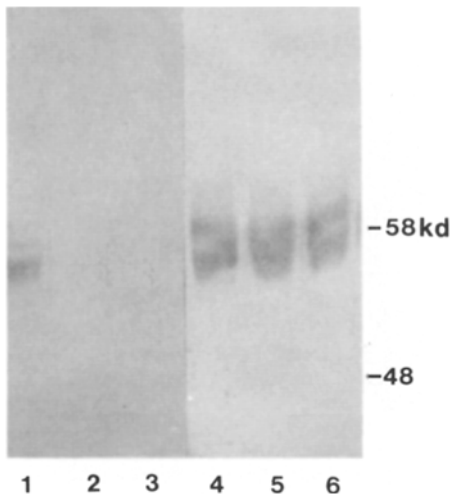
Figure 3. Western blots of U251 (lanes 1 and 4), AS2 (lanes 2 and 5), and AS3 (lanes 3 and 6) cytoskeletal preparations blotted onto nitrocellulose paper and reacted with monoclonal antibodies against GFAP (lanes 1-3) or vimentin (lanes 4-6). The appearance of two bands in the vimentin immunoblot is probably the result of protein degradation.
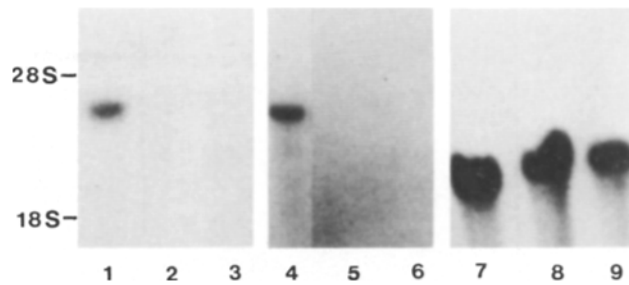

Figure 4. Northern blot analysis of GFAP (lanes 1-6) and vimentin (lanes 7-9) expression in U251 cells (lane 1 and 4), AS2 cells (lane 2 and 5), and AS3 cells (lane 3 and 6) shows the absence of GFAP mRNA in the asGFAP cell-lines, and equivalent hybridization in all cell lines when probed for vimentin (lane 7, U251; lane 8, AS2; lane 9, AS3). Lanes 4-6 are a longer exposure of the first three lanes (3 d vs. overnight).

Table I. Percent of [³H]Thymidine Incorporated

	Neuronal membranes			
	24 h		48 h	
	+	−	+	−
	%			
U251	14	60	10	100
AS2	51	98	8	100
AS3	9	100	11	92

Neuronal cell membranes inhibit the proliferation of the asGFAP and control cell lines. The astrocytic cells were cultured in the presence (+) and absence (−) of neuronal membranes and labeled for 16 h before harvesting and liquid scintillation counting. Percent incorporation = (cpm/cpm of maximal incorporation) × 100. All samples were in duplicate, and SEM was within 15% of mean value.

AS3 cells. The cultures were allowed to grow for 2 d, and then the neurites were visualized with NF2B3, a monoclonal antibody that recognizes the neurofilament heavy subunit. Fig. 5 shows that the extension of neurites in all three types of coculture is comparable. Neurons cultured with HeLa cells quickly disappeared from the cultures, presumably due to cell death, while the HeLa cells continued to proliferate at control levels (data not shown). These results show that GFAP expression does not appear to be required for glial-specific support of neuronal survival and neurite extension. The fact that the AS2 and AS3 cell lines are both able to support neuronal survival and process outgrowth further strengthens the argument that these cell lines had most of the properties of the parent U251 cell line.

AS Cells Fail to Establish Complex, Stable Processes in Response to Neurons

During coculture of astrocytic cells and rodent granule cell neurons, the neurons have been shown to be in close association with the astrocytic cells. The extensive processes of these cells provide a template for neuronal positioning and migration. U251 cells have been shown to be similar to primary cells: they extend complex processes that are intensely immunoreactive with anti-GFAP antibodies (Hatten and Shelanski, 1988; Weinstein et al., 1990). We were able to take advantage of the primate specificity of the anti-MAP4 antibody to distinguish the human astrocytic cells, which were both GFAP positive (U251 cells) and GFAP negative (AS2 and AS3 cells), from rodent neurons and any contaminating primary astrocytes, which were GFAP positive, but

MAP4 negative. Evidence that antibodies to MAP4 and GFAP label the same cells and their processes is shown in Figs. 6, a and b, where the parent U251 cells cocultured with neurons were double labeled with the two antibodies. No differences can be observed in the staining patterns of the two antibodies. The addition of neurons to the AS cells did not result in the induction of GFAP protein expression. In multiple experiments, only a single AS cell was ever observed to double label with the anti-MAP4 antibody and the anti-GFAP antibody, whereas the untransfected U251 cells were always double labeled.

To test the effect of the suppression of GFAP on glial process formation, we established cocultures of U251, AS2, or AS3 cells and primary granule cell neurons. The neurons appeared to be in close association with all of the astrocytic cells lines and grow out processes. Fig. 6, c, e, and f show the phase-contrast images obtained from these cultures, which confirm the presence of neurites previously demonstrated in Fig. 5. However, by anti-MAP4 staining, we could observe a striking difference in the process formation of the
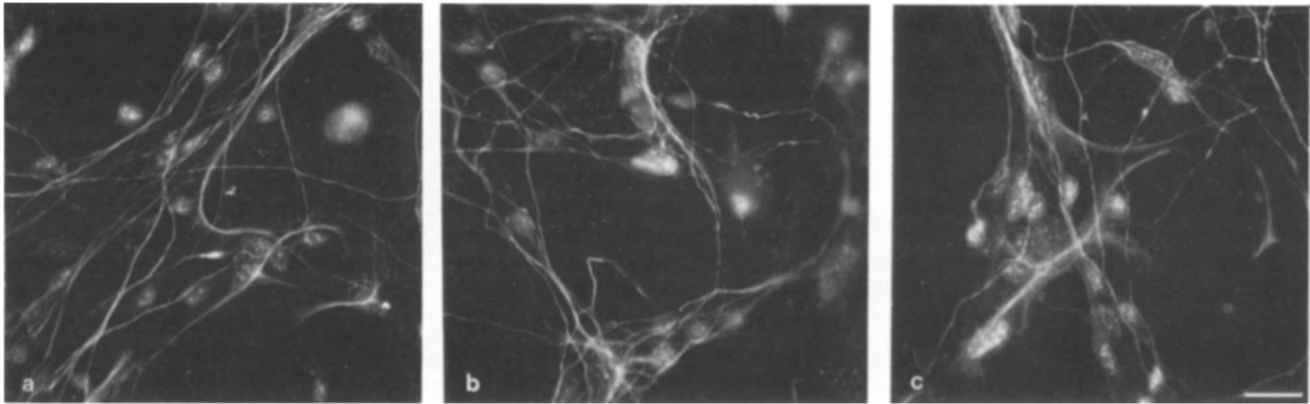
Figure 2. Double-immunofluorescence labeling of the parent U251 cells (a and b), and the stable transfectants AS2 (c and d), and AS3 (e and f) with a polyclonal antiserum against GFAP (a, c, and e) and a monoclonal anti-vimentin antibody (b, d, and f), using fluorescein- and rhodamine-labeled secondary antibodies. The same fields are shown in each pair of micrographs. These results demonstrate that the two U251 lines transfected with the antisense construct have become GFAP negative, but still express vimentin at similar levels as the untransfected cells. Bar, 10 mm.

Figure 5. Interactions of astrocytic cells and neurons. Cocultures of the astrocytic cells and granule cell neurons from neonatal rat cerebellum were analyzed for the ability of the AS cells to support neurite outgrowth. All the astrocytic cell lines supported neurite outgrowth, as assessed by neurite staining with NF2B3, a monoclonal antibody that reacts with the neurofilament heavy subunit (U251, AS2, and AS3, *a–c*, respectively). Bar, 20 mm.

two AS cell lines (Fig. 6, *f* and *h*), when compared with the parent U251 cell line (Fig. 6 *c*). The two AS cell lines were unable to extend the long, complex processes that were typical of cocultures of astrocytes and neurons.

To quantitate the inhibition of glial process formation in the AS2 and AS3 cells, we carried out morphometric analysis of the astrocytic cells in the presence and absence of neurons. Measurements were taken from the tip of a glial process to the middle of the cell soma. As outlined in Table II, there was no significant difference between the U251 cells and the AS cells in the absence of neurons. However, the addition of neurons to the AS cells did not result in these cells undergoing the dramatic changes that are typical of mixed astrocyte–neuronal cultures: the mean change in cell size was small, with an increase in process length of only 14%. The addition of neurons to the U251 cells resulted in an increase of average process length of 389%. It appears unlikely that this difference was due to transfection alone, as the neomycin-transfected cells responded like the untransfected cells.

Discussion

The tissue-specific expression of the intermediate filament proteins suggests that these proteins play a role in the specialized function(s) of the cells in which they are expressed. Because the intermediate filament proteins are part of the cytoskeletal network, it is logical to consider that these proteins are involved in a structural aspect of cell function. In the CNS, astrocytes appear to provide structural support for neuronal migration and positioning. We report here on the suppression of the expression of GFAP in U251 cells by stable transfection with plasmid DNA which constitutively

Table II. Morphometric Analysis of Process Length in Response to Neurons

	− Neurons	+ Neurons
U251 Cells	23.8 ± 7.10	92.8 ± 30
(% increase in length)		(389%)
AS Cells	29.78 ± 8.85	34.2 ± 18
(% increase in length)		(14%)

Astrocytic cells were cultured either in the presence or absence of neurons for two d. The cultures were fixed and stained with an anti-MAP4 antiserum. The length of a given MAP4-positive process was taken as the distance from the ends of the process to the middle of the cell soma ($n > 100$ for each experimental group). Length is expressed in arbitrary units.

transcribed an antisense mRNA of the GFAP gene, driven by a viral promoter. Our studies suggest that GFAP protein, assembled into filaments, is required for astrocytic cells to acquire the structural phenotype that they express in vivo and in vitro.

During the development of the CNS, astrocytes undergo rapid proliferation until the neurons reach their appropriate destination and take up residence near the astrocytes, which then withdraw from the cell-cycle. In vitro studies have shown that cell-to-cell contact is essential for the induction of astrocyte quiescence. Our data do not show any requirements for the expression of GFAP protein for this phenomenon to occur: AS cells are as sensitive to neuronally mediated contact inhibition as the untransfected U251 cells, or control cells transfected with neomycin only. Likewise, we observed no differences between these cell types in their ability to support neuronal survival and neurite extension. Taken together, the antisense suppression of GFAP did not result in the conversion of an astrocytic cell line, U251, to

Figure 6. AS cells no longer form long, stable processes in the presence of neurons. Anti-primate MAP4 antibody recognizes only the human astrocytic cells in mixed neuronal–astrocytic cell cultures. Where GFAP is present, MAP4 immunostaining overlaps with GFAP immunostaining. U251 cells cocultured with neurons were double labeled with anti-GFAP (*a*) and anti-MAP4 (*b*) antibodies, demonstrating the colocalization of these two proteins, and the extensive process formation in response to neurons. *c–h* are cocultures of U251 cells (*c* and *d*), AS2 cells (*e* and *f*), and AS3 cells (*g* and *h*) and neurons. The immunofluorescence images are the astrocytoma cells stained with the anti-MAP4 antibody, demonstrating the absence of significant process formation by the asGFAP cells in response to neurons. All three cell types support neurite outgrowth (*d*, *f*, and *h*). Bar, 10 mm.
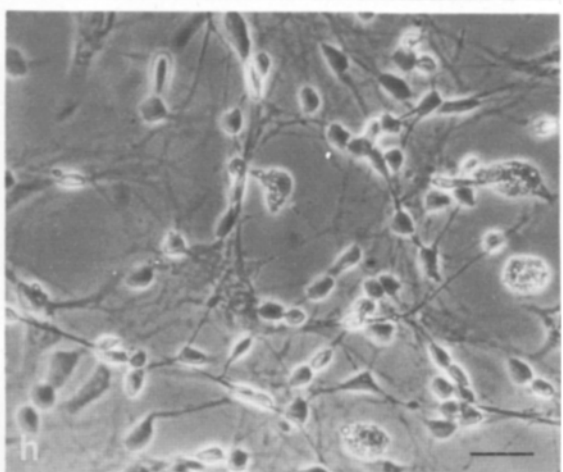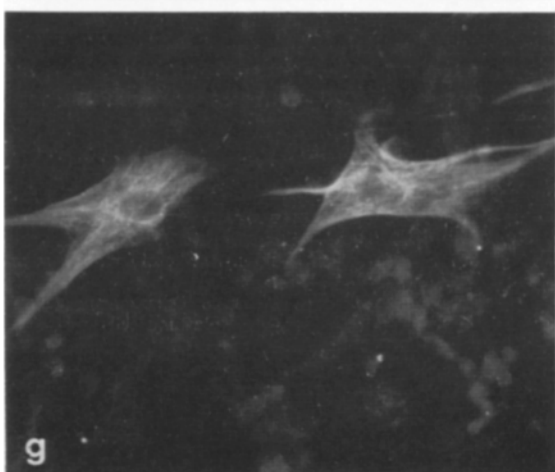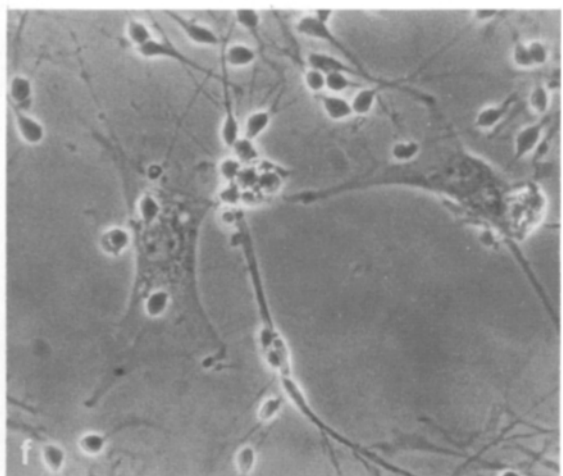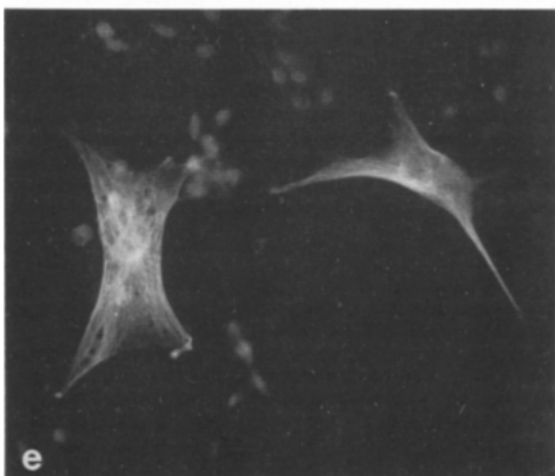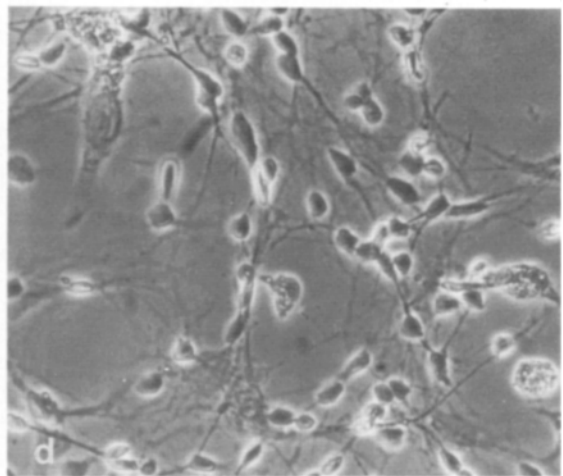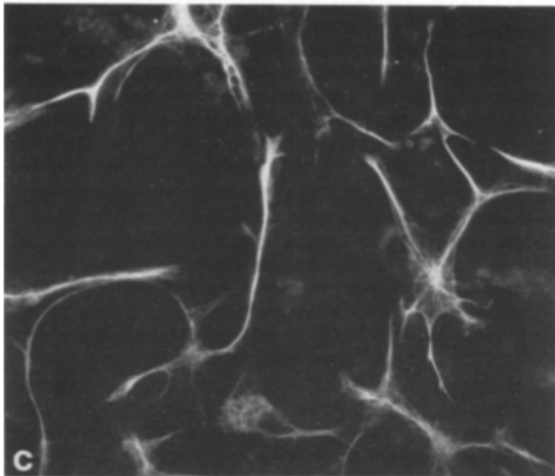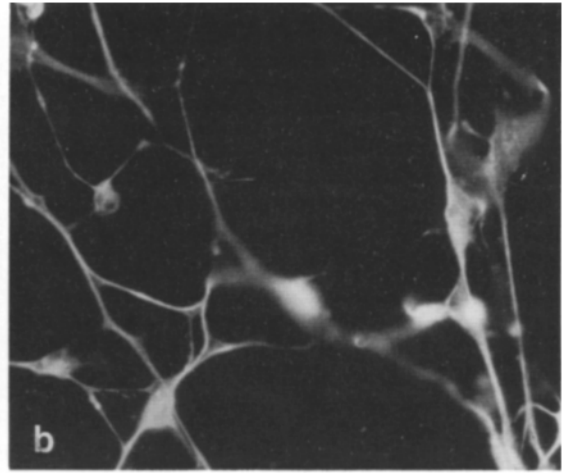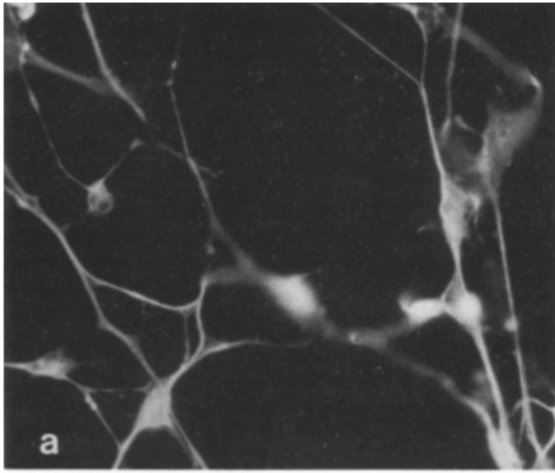
a cell line which was devoid of all astrocyte-associated functions, thus suggesting that the expression of antisense GFAP did not interfere with these significant aspects of the biology of the U251 cells.

During the development of some regions of the CNS, a switch occurs in which vimentin is entirely replaced by GFAP; however, other mature astrocytes continue to express both proteins (Bovolenta et al., 1984; Dahl et al., 1981). Vimentin message is considerably more abundant than GFAP mRNA in U251 cells. This is not unreasonable since most cells in tissue culture express vimentin in high amounts, and the vimentin gene promoter has serum and PDGF response elements (Ferrari et al., 1986). Despite the abundance of vimentin, the structural homologies between vimentin, and GFAP and their coincident expression, our data suggest that vimentin alone is not sufficient to stabilize complex astrocytic processes. When stimulated by neurons, U251 cells, like primary astrocytes, form extensive, GFAP-rich glial processes. In contrast, the AS cell lines undergo only a minor shape change in response to neurons, and do not overcome the antisense suppression of GFAP expression. Northern and Western blot data indicate that these cell types appear to have equivalent amounts of vimentin mRNA and protein, and the cells all have intact vimentin filament networks. Therefore, the differences seen in process extension in response to neurons cannot be due to differences in vimentin expression.

Two lines of evidence support our conclusion that the suppression of GFAP, and the loss of process forming ability in response to neurons in the AS cell lines is a specific result of transfection with the antisense GFAP construct. First, the two stable antisense constructs we have characterized, AS2 and AS3, were the results of separate transfection experiments and they yielded similar results in terms of GFAP suppression and loss of process formation in response to neurons. Second, transfection with an identical plasmid containing only the neomycin resistance gene and its viral promoter did not effect GFAP expression or responsiveness to neurons. Taken together, these controls indicate that the effects we have characterized are not artifacts of transfection alone, but represents a specific suppression of GFAP that results in the loss of process formation in response to neurons.

The data presented in this paper show that removal of a cell-specific intermediate filament, GFAP has a dramatic effect on the formation of glial processes in the presence of neurons even though vimentin expression does not appear to be effected. Both vimentin and GFAP are type III intermediate filaments with relatively high homology (Geisler et al., 1983). The major differences are in the head region, where almost no homology is observed between the two molecules. The tail regions of the type III intermediate filaments show a greater homology to each other than to other types of intermediate filaments, however a considerable amount of variability is still observed. The unique function of GFAP in the formation of stable astrocytic processes could therefore lie in either the variable NH2-terminal or COOH-terminal domains of the GFAP molecule. Our laboratory is currently undertaking studies to investigate the importance of the different domains of the GFAP molecule in its unique function.

The question also remains what effect the addition of neurons may have on the posttranslational modification of GFAP, since our data show that GFAP expression is necessary but not sufficient for astrocytic process formation. U251 cells in the absence of neurons express significant levels of GFAP protein, but do not extend processes. The induction of process formation appears to be a result of a specific interaction between these cells and CNS neurons. It remains to be determined whether this signal leads to posttranslational modification(s) of the glial filament protein, such as phosphorylation, which could allow interactions with other cytoskeletal elements or cell surface components to lead to the formation of the astrocytic processes. Alternatively, it is possible that the neuron triggers the expression or modification of other cellular proteins, which leads to the initiation of astrocytic processes, which in turn are stabilized by the glial specific intermediate filament protein.

In conclusion, we have used an antisense construct of the murine GFAP cDNA to suppress the expression of GFAP in the U251 human astrocytoma cell line. The suppression of GFAP expression results in the inability of the stable transformants to extend processes in response to neurons. The other responses of astrocytes to the addition of neurons, i.e., proliferative arrest and neuronal support are not effected by the loss of GFAP expression. These data support the hypothesis that GFAP functions to stabilize complex glial processes.

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