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Inhibition of GFAP Synthesis by Antisense RNA in Astrocytes

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Glial fibrillary acidic protein (GFAP) accumulation is a prominent feature of astrocytic gliosis. The inhibition or delay in GFAP synthesis might delay scar formation resulting from an insult such as spinal cord injury or central nervous system (CNS) demyelination. The delay in the formation of a physical barrier might allow the neurons and oligodendrocytes to reestablish a functional environment. We delivered antisense GFAP RNA complexed with Lipofectin™ (LF), a cationic liposome, into cerebral astrocytes in culture and tested the feasibility of inhibiting GFAP synthesis. Our results demonstrate that LF facilitated antisense RNA uptake into astrocytes. Astrocytes took up ^{3}H-antisense GFAP RNA alone and reached an equilibrium of 7–8.8 ηg per mg protein after 2.5 hr. When complexed with LF, astrocytes could increase the uptake to 14 ηg per mg protein and the time for reaching this quantity was shortened to 10 min. This uptake level was further enhanced if experiments were carried out in HEPES buffered saline (HBS). All uptake studies were dose- and time-dependent. Dibutyryl cyclic AMP (dBcAMP) is known to induce an increase of GFAP content in cultured astrocytes. We studied the effect of LF/antisense GFAP RNA on the GFAP content in dBcAMP (0.25 mM)-treated astrocytes. Cultures of astrocytes treated with dBcAMP contained almost twice as much GFAP as untreated cultures after 2 days. Similar cultures treated with LF/antisense RNA in HBS did not show an increase but a 30–40% decrease in GFAP content 2 days after treatment. A similar decrease in GFAP content was obtained in cultures grown in a chemically defined medium, another condition known to induce an increase in GFAP content in cultures. This study has demonstrated that antisense GFAP RNA can inhibit GFAP synthesis in astrocytes and may be useful for regulating astrogliosis immediately following CNS injury.

Key words: antisense RNA, astrocytes, cultures, GFAP, GFAP synthesis, liposomes, lipofection

INTRODUCTION

Glial fibrillary acidic protein (GFAP), the major component of the intermediate filament in differentiated astrocytes (Eng et al., 1971; Eng, 1985), is extensively synthesized within and adjacent to the site of injury (Condorelli et al., 1990; Eng, 1988a; Hozumi et al., 1990; Vijayan et al., 1990). This stereotypic reaction of astrocytes occurs in spinal cord injury and central nervous system diseases such as multiple sclerosis, Alzheimer's disease, Creutzfeldt-Jakob disease, and Huntington's disease (Eng, 1988a,b; Reier, 1986). Such gliotic responses may interfere with the function of residual neuronal circuits, by preventing remyelination, or by inhibiting axonal regeneration (Eng et al., 1987; Reier and Houle, 1988; Stensaas et al., 1987). Inhibition of GFAP synthesis might delay the gliotic reaction and the formation of a physical barrier, thus allowing neurons and oligodendrocytes to reestablish a functional environment.

This study tested the feasibility of inhibiting GFAP synthesis with antisense RNA to GFAP prepared from long cDNA sequences of GFAP mRNA. Use of antisense RNA offers a method for manipulation of gene expression (Dolnick, 1990; Hélène and Toulmé, 1990; Marcus-Sekura, 1988; Strickland et al., 1988; Takayama and Inouye, 1990). In this study, the antisense RNA to GFAP was introduced into cultured astrocytes with Lipofectin® Reagent (LF), a cationic liposome (Felgner et al., 1987; Eng et al., 1990; Yu et al., 1991). Liposomes are single-walled lipidic vesicles that have been used to deliver active substances into living cells (Mannino and Gould-Fogente, 1988; Nicolau and Cudd, 1989; Papahadjopoulous, 1988).

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Cultures of rodent cerebral astrocytes were used as a model for studying effects of antisense GFAP RNA on GFAP synthesis in astrocytes. Astrocyte cultures present several advantages to this study. They are easily accessible structures, contain no neuronal cell bodies, and the effects of treatment can be easily quantitated. The biochemistry and development of astrocytes in cultures have been extensively studied by others (Hertz, 1982; Juurlink and Hertz, 1985; Kimelberg, 1983). The GFAP content in these cultures can be manipulated by chemicals such as dibutyryl cyclic adenosine monophosphate (dBcAMP) (Chiu and Goldman, 1985; Goldman and Chiu, 1984; Hertz et al., 1978; Shafit-Zagardo et al., 1988) and chemical defined medium (Morrison et al., 1985). A preliminary report of this study has been published (Eng et al., 1991).

MATERIALS AND METHODS

Culture Preparation

The preparation of cultures of astrocytes from cortices of new-born Sprague-Dawley rats (VAMC animal facility, Palo Alto, CA, USA) has been described previously (Yu et al., 1989). The neopallium, i.e., the portion of cortex dorsolateral to the lateral ventricles, was obtained aseptically from the brain. The neopallia freed of meninges were cut into small cubes (<1 mm^3) in a modified Dulbecco's modified Eagle medium (DMEM) (JRH Biosciences, Lenexa, KS, USA). The tissue was disrupted by vortex mixing for 1 min and the suspension was passed through two sterile nylon Nitex sieves (L. and S.H. Thompson, Ontario, Canada) with pore sizes of 80 μm and 10 μm. A volume of cell suspension with about 3×10^5 cells was placed in a 35-mm Falcon tissue culture dish (Becton Dickinson and Co., NJ, USA). Fresh DMEM supplemented with 20% fetal calf serum (FCS) (HyClone Laboratories, UT, USA) was added to yield a final volume of 2 ml. All cultures were incubated at 37°C in a 95%:5% (vol/vol) mixture of atmospheric air and CO_2 with 95% humidity. The culture medium was changed 3 days after seeding and subsequently two times per week with DMEM containing 10% FCS. Cultures were at least 4 weeks old when used for the experiment.

Antisense GFAP RNA Preparation

Antisense GFAP RNA was prepared in the authors' laboratory. Hamster GFAP cDNA (Scr-1) sequence with 1K bp inserted in pSP65 in EcoR1 site (a gift from Dr. Ashley T. Haase) (Wietgrefe et al., 1985; Diedrich et al., 1987) was linearized by BamHI and then used as a template in an in vitro run-off transcription reaction to produce large quantities of RNA by using Promega Biotec Riboprobe Transcription System Kit (Melton et al., 1984). To prepare the complex, antisense RNA and 11 μg of LF (BRL Life Technologies, Inc., Gaithersburg, MD, USA) were mixed in a polystyrene tube for 15 min at ambient temperature (Eng et al., 1990, 1991).

Antisense RNA Uptake

Antisense RNA covalently linked to a photo activatable form of biotin (Forster et al., 1985) was used as a probe to study its uptake into astrocytes. Cultures grown on Thermanox™ tissue culture cover slips (E&K Scientific Products, Seratoga, CA, USA) were washed three times with 37°C serum-free DMEM before being incubated in the same DMEM containing LF/biotinylated antisense RNA. The incubation was terminated by washing the cultures three times with 0.9% NaCl and fixed with 70% EtOH in 0.3 M NaCl for 30 min. The fixed cultures were incubated with rabbit antibody to biotin (ENZO Biochemical, NY, USA), followed by immunoperoxidase staining as described by Sternberger et al. (1970) and finally counterstained with hematoxylin.

For the uptake of LF/^{3}H-antisense RNA, cultures were washed with serum-free DMEM or HEPES buffered saline (HBS) twice before LF/^{3}H-antisense RNA complex was added. ^{3}H-Antisense RNA (2×10^5 cpm per μg) was synthesized by Promega Biotec Riboprobe Transcription System Kit (Melton et al., 1984). The cells were rapidly washed twice with ice-cold DMEM or HBS and dissolved in 0.5 ml of 2 N NaOH at the end of the uptake experiments. The radioactivity and protein content in the cell extracts were determined in a Packard Tri-carb 460C liquid scintillation counter and by Lowry assay (Lowry et al., 1951), respectively. The uptake was calculated from the radioactivity per milligram of protein and the specific activity in the incubation medium. The uptake efficiency was expressed as the percentage of the antisense RNA uptake to the total amount of antisense RNA added to the culture.

Antisense RNA Effects on dBcAMP-Treated Cultures

To study effects of antisense RNA on GFAP synthesis in cultures of astrocytes, confluent cultures were exposed to dBcAMP (0.25 mM) for 2 days before transfection with antisense GRAP RNA or LF complex in HBS. Cultures were incubated with the complex (6 μg antisense RNA and 11 μg of LF) for 3 hr before fresh culture medium, containing dBcAMP and with or without FCS, was added. Both control and transfected cultures were fed with fresh medium after 16 hr. Cultures were harvested by addition of 1 ml of 1% SDS in 50 mM phosphate buffer (pH 8.0) (Morrison et al., 1985) 1, 2, 3, 5, or 7 days after transfection. GFAP content was measured by a solid phase enzyme-linked immunosorbent assay (ELISA) (Eng et al., 1986). The change in GFAP content was further investigated by one-dimen-

Fig. 1. Photomicrograph of immunostained astrocytes exposed to LF/biotinylated antisense GFAP RNA. Cells were transfected with the complex for 30 min. The fixed cultures were incubated with rabbit antibody to biotin, followed by immunoperoxidase staining, and finally counterstained with hematoxylin before mounting. Uptake of the LF/antisense RNA complex by the astrocytes is indicated by the coloring of the cell bodies and processes (arrows). Bar = 40 μm.

sional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels by the method of Laemmli (1970), followed by transfer to nitrocellulose membranes according Towbin et al. (1979) and immunoperoxidase staining with antibody to GFAP (Eng and DeArmond, 1983; Sternberger et al., 1970).

RESULTS

Antisense RNA Uptake

Astrocytes in cultures exposed to biotinylated antisense GFAP RNA showed positive reaction to antibody to biotin (Fig. 1). The staining was observed as early as 30 min exposure to the complex. No staining was observed in cultures exposed to LF or biotinylated antisense RNA alone (data not shown). This indicated that LF facilitated the entry of antisense GFAP RNA into astrocytes.

Figure 2 compares the uptake of antisense GFAP RNA (0.25 μg per culture) alone to LF complex in DMEM or HBS. ^{3}H-Antisense RNA entered astrocytes even in the absence of LF. Between 2.5 to 6 hr of exposure, astrocytes took up 7–8.8 ηg antisense RNA per mg protein, with an uptake efficiency of 3.4%. The uptake was accelerated and enhanced when antisense RNA was complexed with LF. In DMEM, the time for astrocytes to take up a similar amount of antisense RNA in LF as antisense alone was shortened to 10 min. The total uptake was also elevated to 14 ηg per mg protein in the 6-hr experimental period, with an uptake efficiency of 5.6%. When incubated in HBS, the amount of antisense RNA that entered the astrocytes almost doubled (> 20 ηg per mg protein) in 10 min of transfection, and the uptake efficiency was increased to almost 10%.

The uptake of antisense RNA was increased when the dose of antisense RNA was elevated (Fig. 3). All experiments were measured at 1 hr of incubation. The uptake of antisense RNA alone was low with an uptake efficiency of about 1.5%, despite the doses. With LF as a mediator, the uptake was higher than antisense RNA alone. In HBS, the uptake efficiency averaged 7.6%, higher than 2.9% in DMEM. The uptake efficiency remained the same at doses up to 15 μg. Examination of Figures 2 and 3 shows that the initial uptake rates in HBS were always the highest, and reached an optimal efficiency within 10 min. Uptake of the complex in DMEM was always lower than in HBS, but was higher than for the antisense RNA alone. It took at least 6 hr to achieve optimal efficiency under both conditions. The data also indicated that the 11 μg of LF in the complex could introduce a large amount of antisense RNA into astrocytes. Based on these observations, dBcAMP-treated astrocytes was transfected with LF/antisense GFAP RNA in HBS and the changes in GFAP content was determined.

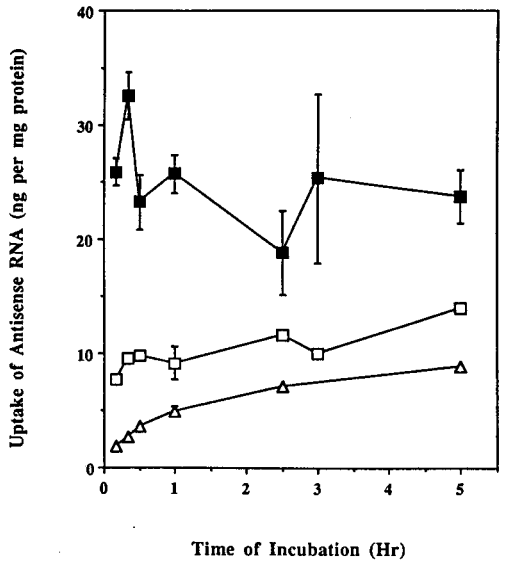

Fig. 2. Uptake of ^{3}H-antisense GFAP RNA as a function of time of exposure to LF/antisense RNA complex in culture of astrocytes. The concentration of antisense RNA was 0.25 μg per culture. One milliliter of complex contained 11 μg of LF. Uptake was measured for antisense RNA alone in DMEM (△); and for LF/antisense RNA in DMEM (□) and in HBS (■). Each point was an average of three to ten experiments and SEM values are shown by vertical bars if they extend beyond the symbols.

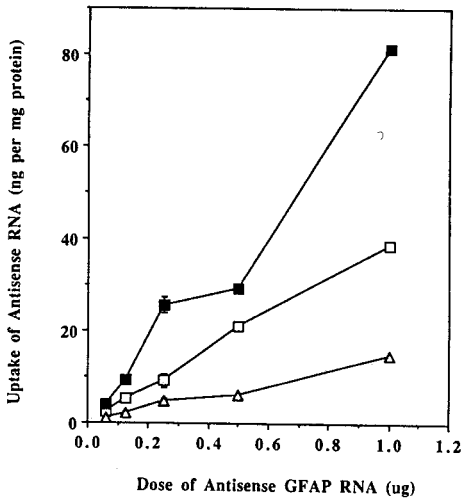

Fig. 3. Uptakes of antisense GFAP RNA as a function of the dose of antisense GFAP RNA in the LF/RNA complex. One milliliter of complex contained 11 μg of LF. Uptake was measured for antisense GFAP RNA in DMEM (△); and for LF/antisense GFAP RNA complex in normal DMEM (□) and in HBS (■). Time of exposure was 1 hr. Each point was an average of three to ten experiments and SEM values are shown by vertical bars if they extend beyond the symbols.

Effects of Antisense RNA on GFAP Synthesis in dBcAMP-Treated Culture

Astrocytes in cultures treated with 0.25 mM of dBcAMP showed an increase in GFAP content (Fig. 4). The GFAP content in astrocytes at day 0 (i.e., immediately before transfection) was used as the control and the changes were expressed as the percent increase or decrease with respect to this value. In serum-free medium, an immediate increase in GFAP content induced by dBcAMP was observed (Fig. 4B). The increase reached about 160% on day 3, then remained at this level for the rest of the experiment (Fig. 4B). In serum-containing medium, the stimulation by dBcAMP was delayed and a gradual increase was observed on day 2 (Fig. 4A). The GFAP content under this condition also reached a level greater than 160% of the control at the end of the experiment.

Although dBcAMP was in the culture medium, all transfected cultures showed a decrease in GFAP content (Fig. 4A,B). The decrease was more pronounced in cultures maintained in serum-free medium (Fig. 4B). One day after transfection, a 30% decrease in GFAP content was achieved. The content was further decreased to less than 50% of its original level after the third day of transfection. In serum-containing medium, a significant decrease in GFAP content of 30% was observed three days after transfection (Fig. 4A). Similar results were obtained in three repetitions of this experiment. The GFAP content began to return gradually to the day 0 level 5 days after transfection in some cultures but never reached a level comparable to the corresponding nontransfected sister cultures. A similar result was observed in cultures of astrocytes exposed to a chemical defined medium (data not shown), a condition also known to increase the GFAP content of astrocytes in culture (Morrison et al., 1985). Antisense RNA alone and complexes with lower doses of antisense RNA (<3 μg per culture) did not induce any observable effect on the GFAP content.

The inhibition was further confirmed by separating the total cellular protein with SDS-PAGE, transblotting to nitrocellulose, and estimating the changes in GFAP content by immunoperoxidase staining with antibody to GFAP. Results indicated that the GFAP content was clearly decreased in cultures 3 days after transfection (Fig. 5). The content gradually returned to day 0 level but remained lower than comparable nontransfected culture. Occasionally, there were the appearance of lower molecular weight bands which might be the degradation products of GFAP.

DISCUSSION

Interest in the use of antisense RNA for the study of gene expression and regulation has increased dramati-

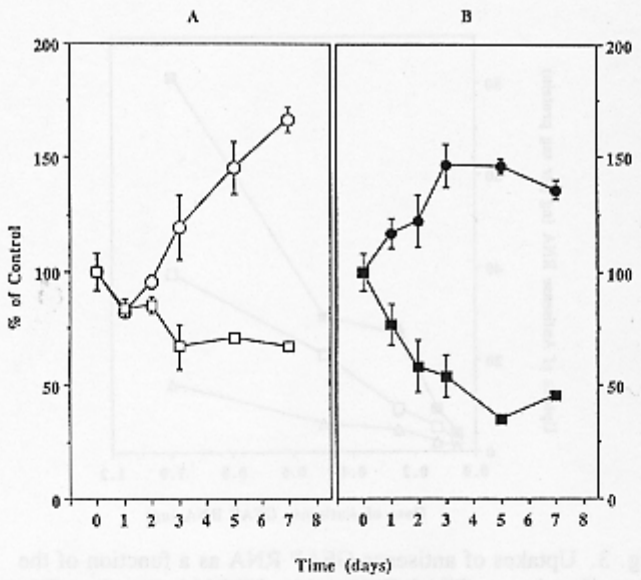

Fig. 4. Effect of LF/antisense GFAP RNA transfection on the GFAP content of astrocytes in cultures. Cultures were incubated in DMEM containing 0.25 mM dBcAMP and 10% serum for 2 days before transfected for 3 hr in HBS. Then all cultures were incubated in DMEM contained dBcAMP supplemented with (A) or without (B) serum. GFAP content was quantitated by ELISA. The GFAP contents in transfected (□; ■) and non-transfected (○; ●) cultures were expressed as the percentage of the value at day 0. One milliliter of complex contained 6 μg antisense RNA and 11 μg of LF. Each point was average of four measurements and SEM values are shown by vertical bars if they extend beyond the symbols.

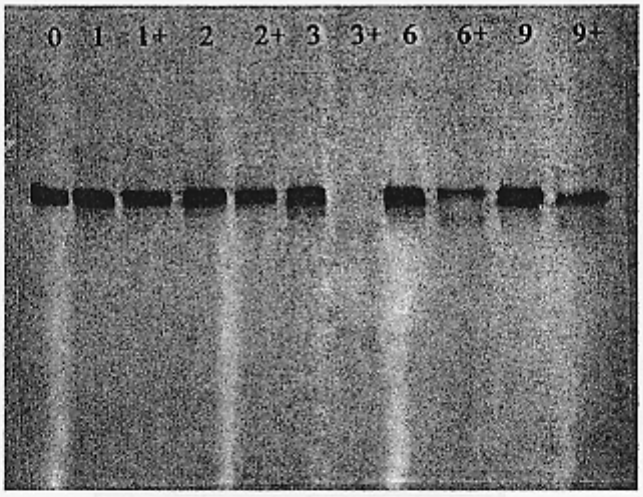

Fig. 5. The effect of antisense GFAP RNA on the synthesis of GFAP in culture of astrocytes treated with dBcAMP is shown by one-dimensional SDS-PAGE in 10% acrylamide gels with 20 μg total extracted protein per lane, transblotting to nitrocellulose, and estimating the changes in GFAP content by immunoperoxidase staining with antibody to GFAP. Cultures treated with dBcAMP alone are labeled 1, 2, 3, 6, and 9, corresponding to their days of treatment; dBcAMP and antisense RNA treated cultures are labeled with (+). Transfection was done with 6 μg antisense RNA and 11 μg of LF in HBS for 3 hr. All cultures were incubated in medium with dBcAMP and serum throughout the experiment.

cally in the past two years. In this study, astrocytes can be transfected with antisense RNA targeted to mRNA of GFAP. The transfection was more efficient with LF and in HBS. Transfected astrocytes showed a decrease in GFAP content.

The inhibitory effect of antisense RNA on GFAP synthesis lasted for at least 3 days and, in some cases, began to fade after 5 days. The antisense RNA presumably functions in the cytoplasm and most likely inhibits gene expression of GFAP by hybridization arrest of translation. The transient nature of the inhibition may be due to limited stability of the antisense RNA in the medium and their duplex with its complementary GFAP mRNA within the cells. This might explain our observation that intracellular transfected nucleic acids disappeared very quickly after the removal of transfection medium (Eng et al., 1990, 1991). The long-lasting but ultimately transient effect of the antisense RNA (Fig. 4) might be composed of two phases of action. The first phase is a depletion of the sense RNA (a pure antisense effect), and the second phase is a slow replenishment of new sense RNA by transcription.

Usually, inhibition is effective only with a high ratio of antisense:sense genes, but sometimes inhibitory effects can be quite dramatic even with a 1:1 ratio (Rubenstein et al., 1984). In this study, inhibition was not detected in culture transfected with antisense RNA alone, nor with low doses (< 3 μg) of antisense RNA in LF complex. Weak inhibition was observed in some cultures transfected with a complex that contained 3 μg antisense RNA. Only with complexes containing 6 μg of antisense RNA was the inhibition of GFAP synthesis significant and reproducible. With a 7.6% uptake efficiency, 450 ηg of antisense RNA entered the culture. Based on our previous findings (Eng et al., 1990, 1991), at least one-sixth of this antisense RNA (75 ηg) would be available to hybridize with the sense GFAP mRNA. A 35-mm astrocytic culture contains about 7.5 μg RNA (unpublished data) and about 1–5% of this is mRNA; the ratio of antisense to the sense GFAP mRNA (only a small percentage of the mRNA) would be around 100:1. This ratio is relatively high and may be improved in the future by modification of mRNA penetration through cell membranes, specificity to the target gene, and, most important of all, resistance to nucleases (Agris et al., 1986; Agrawal et al., 1989; Morvan et al., 1987; Smith et al., 1986; Thuong et al., 1987). The latter could

be achieved by linking the 3′ end of the antisense RNA to an additional phosphorothioate base (Agrawal et al., 1989; Ning et al., 1991).

The poor absorption of unmodified oligomer by cells limited their use in vitro and in vivo. This is especially true for the large size antisense RNA used in this study. Lipofectin™ Reagent, a cationic liposome, facilitates fusion of nucleic acid with the plasma membrane of tissue culture cells, resulting in both uptake and expression of the nucleic acid (Felgner et al., 1987). It had previously been shown that 11 μg LF is a nontoxic dose that facilitated the uptake of DNA by astrocytes in culture (Eng et al., 1990, 1991). In this study, the same liposome preparation also mediated the entry of antisense RNA to astrocytes (Fig. 2, 3). The uptake was further enhanced in HBS, a simpler buffer compared to the DMEM. The complex of salts and nutrients in the DMEM might have obstructed the fusion of LF with the cell membrane, which resulted in a lower uptake (Fig. 2, 3). The antisense inhibition was less in serum-containing medium (Fig. 4). This may not be a direct effect of serum on the expression of the antisense gene, but due to a reduction in the availability of the complex to the cells. It is known that serum proteins can bind to many different types of compounds. The binding of LF/antisense RNA to serum might reduce the amount of complex entering the cells. Furthermore, serum nucleases may degrade the antisense RNA in the complex before hybridization occurs.

Very little is known about possible extracellular signals regulating GFAP gene expression and the rapid GFAP synthesis during reactive astrogliosis. Some clues on cAMP and protein kinase C involvement come from studies in cell culture models (Condorelli et al., 1990; Shafit-Zagardo et al., 1988). Several studies have shown that the rate of GFAP synthesis and accumulation can be manipulated in primary astrocyte cultures (Chiu and Goldman, 1985; Goldman and Chiu, 1984; Hertz et al., 1978). Our observation of an increase in GFAP content in astrocyte cultures induced by dBcAMP agrees with these reports. A decrease in GFAP mRNA levels in the presence of serum has also been reported (Shafit-Zagardo et al., 1988). The delay in GFAP response to dBcAMP treatment in our serum-containing cultures might be explained by a serum component that inhibits the cellular response to cAMP (Kessler et al., 1986).

The method for controlling gene expression with antisense nucleic acid has provided a powerful tool in identifying genes, characterizing gene functions, controlling virus and parasite infections, and manipulating metabolic pathways (Agris et al., 1986; LeDoan et al., 1989; Morvon et al., 1987; Smith et al., 1986; Thuong et al., 1987). We have successfully inhibited GFAP synthesis in cultured astrocytes with antisense RNA to GFAP. The transfection was optimized with LF and in HBS. In this culture model, the inhibitory effect of antisense RNA on GFAP synthesis was significant. In comparing transfected cultures with corresponding nontransfected cultures, the actual inhibition was much higher than the values stated in the results section. The true inhibition values should also include that amount of increase in GFAP content stimulated by dBcAMP, which was also suppressed by the antisense RNA. An increase in biosynthesis of GFAP is a characteristic of gliosis (Eng, 1988a,b). Control of gliosis may, in turn, be linked to GFAP synthesis. Inhibition of GFAP synthesis immediately following injury might delay astrogliosis. Thus, antisense nucleic acid treatment may be a tool for modulation of astrogliosis to promote healing and functional recovery of neuronal pathways.

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