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Serum-free mouse embryo (SFME) cells are derived in medium in which serum is replaced with growth factors and other supplements. They display unusual properties. They do not lose proliferative potential or show gross chromosomal aberration upon extended culture, they depend on epidermal growth factor (EGF) for survival, and are reversibly growth inhibited by plasma and serum. In the presence of transforming growth factor beta (TGF-ß) SFME cells express the astrocyte marker, glial fibrillary acidic protein (GFAP).

The growth inhibitory activity of human plasma on serum-free mouse embryo cells was investigated. Human plasma did not inhibit SFME cells transformed with the human Ha-ras oncogene. The activity was

present in delipidated plasma and was not dialyzable against 1 M acetic acid. The activity could be precipitated by methanol, bound to concanavalin A-agarose and was retarded by Sephadex G-50 in 200 mM acetic acid. A fifty to hundred fold purification was achieved, although the differential inhibition of untransformed versus transformed cells was lost in the course of the purification.

Using the technique of differential screening of a cDNA library a calf serum- and TGF-ß-regulated mRNA species was identified in SFME cells. This mRNA was approximately 8.5 kilobases in size and brain-specific. Picomolar quantities of TGF-ß caused an increase of this message in SFME cells within four hours. This increase was reversed when TGF-ß was removed from the culture medium.

Studies on the Growth Inhibition and Differentiation of Serum-free Mouse Embryo (SFME) Cells

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Chapter I

INTRODUCTION

One of the major questions of biology is how do growth control and differentiation function in multicellular organisms. Because of the complexity of the task one tries to gain insights by using model systems. Cells growing in vitro probably provide the most widely used model systems in biology. A major focus of animal cell biology is the question how do factors outside of the cell influence growth and differentiation of the cell. Some of the most important substances in this respect are the peptide growth factors. In the studies presented here we use serum-free derived mouse embryo cells (SFME) to investigate growth control and differentiation in vitro.

Transforming growth factor beta (TGF-ß). Peptide growth factors are major regulators of development in animals. They begin to act very early in embryonic

development. (For review on peptide growth factors in early development: Mercola et al., 1988; David et al., 1990; Slack, 1990; Marx, 1991). The term "peptide growth factor" is an incomplete and misleading description of substances that may actually inhibit growth and/or cause differentiation in vivo and in vitro depending on cell type, growth conditions etc. (Sporn et al., 1988).

Peptide growth factors form gene families of closely related factors with structural and functional similarities (Mercola et al., 1988). One of the most intriguing of those families is the TGF-ß family. The first member of the family, now called TGF-ß1, was initially discovered and purified as a factor which induces phenotypic transformation (morphological transformation and anchorage independent growth) of a nonmalignant fibroblastic cell line (Roberts et al., 1983). To the TGF-ß gene superfamily belong the TGF-ß1 through 5, the inhibins, activins, the decapentaplegic gene, and the Müllerian inhibiting substance (Massagué et al., 1990). TGF-ß1 is a 25,000 molecular weight, disulfide-linked homodimer. Each chain is synthesized as the C-terminal domain of a larger precursor that

contains a signal sequence for translocation across the endoplasmatic reticulum (Miyazono et al., 1988). TGF-ß1 has been purified from human and porcine blood platelets (Assoian et al., 1983), from human placenta (Frolick et al., 1983), and bovine kidney (Roberts et al., 1983). The most studied TGF-ßs are TGF-ß1, 2, and 3. In cell culture they often act similarily (Cheifetz et al., 1987; Graycar et al., 1989), but sometimes show marked differences (Ohta et al., 1987; Ottmann et al., 1988; Tsunawaki et al., 1988; Jennings et al., 1988). TGF-ß causes a multitude of effects <u>in vitro</u> depending on cell type and culture conditions. Although TGF-ß is growth stimulatory for some cell types, in many cases it is a strong growth inhibitor, especially for cells of epithelial origin, but also for T-and B-lymphocytes (Miyazaki et al., 1989). TGF-ß controls cell adhesion, extracellular matrix protein expression, pericellular proteolysis, and cell adhesion receptors. It can cause or inhibit cell differentiation, depending on cell type. TGF-ß is thought to be involved in embryogenesis, immunosuppression, fibrosis, and oncogenesis (for review on TGF-ß: Massagué et al., 1990).

<u>Serum-free cell culture</u>. The discovery and

widespread availability of peptide growth factors such as epidermal growth factor (EGF) enabled the development of serum-free cell culture, where serum is replaced by growth factors, binding proteins (e.g. transferrin and albumin), attachment proteins (e.g. fibronectin) and other proteins (Barnes et al., 1987; Barnes et al., 1980). Serum-free cell culture in turn is a powerful tool to study the action of peptide growth factors. Those studies reveal how such factors influence growth and differentiation of various cell types, thereby providing models of what may happen in vivo.

One of the insights gained by serum-free cell culture is that serum can inhibit growth of various cell types (Ambesi-Impiambato et al., 1980; Loo et al.,1987). The inhibition may be a specific response to some hormone or the result of some unspecific cytotoxic effect. Possible inhibitory activities present in serum are hormones or hormone-like substances such as TGF-ß (Miyazaki et al., 1988), interferons (Chen et al., 1988) , interleukins (Saneto et al., 1986), 3,5,3'-l-triodothyronine and hydrocortisone (Loo et al., 1989), tumor necrosis factors (Sugarman et al., 1985) and others. Lipoproteins have been reported to be

inhibitory (Ito et al., 1982). Proteases and protease inhibitors may also be inhibitory. Serum amyloid A protein has been reported to inhibit growth of several cell types in culture (Peristeris et al., 1988).

Serum-free mouse embryo cells. If one wants to study the effects of growth factors in cell culture, one has usually two choices: one can use primary culture of freshly obtained cells from an animal or one can use established cell lines such as Balb 3T3, Hela, or PC 12. Primary culture has the advantage that the cells are closest to what is present in the whole animal, but often the cell composition is heterogeneous, the cells are not well characterized and their properties change during culture. Established cell lines are mostly homogeneous, in many respects well characterized, and stable, but generally they are genetically altered, showing gross chromosomal aberrations.

Serum-free cell culture made it possible to establish a mouse embryo cell line that combines the advantages of established cell lines and of primary cultures. Serum-free mouse embryo cells (SFME) are derived in a rich basal nutrient formulation supplemented with EGF, insulin, transferrin, high

density lipoprotein (HDL), and fibronectin (Loo et al., 1987). They are homogeneous and stable in long term culture and do not show gross chromosomal aberrations. They do not lose their proliferative potential during establishment and culture, thus they show no signs of senescence or growth crisis as in classical serum-containing mouse embryo cell culture. They depend on EGF for survival and are reversibly growth inhibited by serum or platelet-free plasma. Treatment of these cells with serum or TGF-ß causes the appearance of glial fibrillary acidic protein (GFAP) (Sakai et al., 1990). GFAP is a specific marker for astroglial cells, which are a major cell type found in the central nervous system (CNS). These findings suggest that SFME cells may provide insights in astrocyte precursor growth regulation and astrocyte differentiation.

Astrocytes. Astrocytes are the most abundant cells in the CNS, outnumbering neurons by about 10:1. The astrocyte lineage represents actually a family of cell types that share biochemical and morphological traits but diverge in functional capabilities. Even though early on astrocytes were recognized as specific elements of the CNS, their diversity of functions have

only recently been revealed and are still subject of intense research (Kimelberg et al., 1989; Frohman et al., 1989; Prochiantz et al., 1988). The defining characteristic of astrocytes is the expression of specific intermediate filaments whose major structural component is the 49,000 molecular weight protein GFAP (Eng et al., 1971). An important behavior of astrocytes in response to pathological situations is a chronic hyperplastic change. It is characterized primarily by deposition of GFAP fibrills and termed gliosis. It occurs as a response to CNS injury.

Astrocytes in cell culture. Astrocytes have been studied extensively in culture. This has been facilitated greatly by the fact that one can easily obtain essentially pure astrocyte primary cultures (Hertz et al., 1982). However, the reported properties of the cultured astrocytes often differ markedly (Juurlink et al., 1985). This may be due to differences in the source of the cells with respect to species or region of the CNS, dissociation procedures, culture medium, type and/or concentration of serum used in culture, if any (Juurlink et al., 1985). The properties of the cells may change with time in culture.

The heterogeneity of astrocytes is illustrated in the work of M.C. Raff and coworkers (for review: Lillien et al., 1990). They showed that rat optic nerve primary cell culture contains two morphologically, biochemically and immunohistochemically distinct types of astrocytes: type-1 astrocytes and type-2 astrocytes (Raff et al., 1983). The studies of M. C. Raff and coworkers show how these two types of astrocytes interact and interdepend. Their studies are probably the most comprehensive investigations of astrocyte differentiation in vitro. Raff and coworkers showed that type-2 astrocytes develop from progenitor cells that have the capability to give rise to oligodendrocytes or type-2 astrocytes. In a serum-free culture medium the progenitor cell is committed to the oligodentrocyte lineage. But in the presence of fetal bovine serum (FBS), optic nerve extract or ciliary neuronotrophic factor (CNTF) they develop to GFAP positive type-2 astrocytes (Hughes et al., 1988). Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and CNTF inhibit the differentiation of progenitor cells into oligodentrocytes (Noble et al., 1988). PDGF promotes division and motility and inhibits premature

differentiation of the oligodentrocyte/type-2 astrocyte progenitor cell.

Astrocyte cell lines, derived from tumors or otherwise transformed cells have been used to study astrocyte differentiation. But these cells are often genetically abnormal and probably reflect astrocytic properties in vivo to a lesser extent (Bressler et al., 1992, Hertz et al., 1985).

Chapter II

CHARACTERIZATION OF HUMAN PLASMA GROWTH INHIBITORY ACTIVITY ON SERUM-FREE MOUSE EMBRYO (SFME) CELLS

SUMMARY

The growth inhibitory activity of human plasma on serum-free mouse embryo cells was investigated. Human plasma does not inhibit SFME cells transformed with the human Ha-ras oncogene. The activity was present in delipidated plasma and was not dialyzable against 1 M acetic acid. The activity could be precipitated by methanol, bound to concanavalin A-agarose and was retarded by Sephadex G-50 in 200 mM acetic acid. A fifty to one-hundred fold purification was achieved, although the differential inhibition of untransformed versus transformed cells was lost in the course of the purification.

INTRODUCTION

SFME cells are mouse embryo cells derived in serum-free basal nutrient medium supplemented with epidermal growth factor (EGF), insulin, transferrin, high density lipoprotein (HDL), selenium, and fibronectin (Loo et al., 1987). These cells exhibit several unusual characteristics. (i) They do not exhibit growth crisis or gross chromosomal aberration even in long term culture (Loo et al., 1987). (ii) In the presence of transforming growth factor beta (TGF-ß) SFME cells express the astrocyte marker, glial fibrillary acidic protein (GFAP) (Sakai et al., 1990). (iii) SFME cells are dependent on EGF for survival and (iv) are reversibly growth inhibited by serum and platelet-free plasma (Loo et al., 1987). (v) Transformation of these cells with the ras or neu oncogenes releases the cells from the growth inhibition by serum/plasma and the EGF requirement for survival (Shirahata et al., 1990).

Culture of the SFME cells in the presence of serum causes a rapid growth inhibition, followed after several weeks of culture by the outgrowth of rare, abnormal,

hyperploid variants (Rawson et al., 1991). Exposure of the cells to calf serum for as little as 8 h causes a near maximal effect (Rawson et al., 1991). The behavior of SFME cells in serum is probably not simply a toxic response because it is reversible (Loo et al., 1987; Rawson et al., 1991) and the cells arrest in the G 1 phase of the cell cycle in the presence of 10 % calf serum (Rawson et al., 1991). In medium with 10 % serum, incorporation of thymidine into DNA and the activity of thymidine kinase (an S-phase dependent enzyme) is reduced, while incorporation of amino acids into protein is not (Rawson et al., 1991). These results suggest that the growth inhibition may be a response of the SFME cells to a physiological mediator.

Glucocorticoid and thyroid hormones may contribute to the inhibitory activity of serum, but Loo et al. (1990) have shown that at least one additional factor must be involved. Heat inactivation of serum or plasma (56°C,30 minutes) does not diminish the growth inhibitory activity. Sera from a variety of species, including calf, adult, and fetal bovine sera, human serum and plasma, and mouse serum are inhibitory (Loo et al., 1987). Calf serum is of somewhat higher inhibitory

activity than calf plasma (Loo et al., 1987).

TGF-ß is a strong inhibitor for many types of cultured cells, especially those of epithelial origin (Miyazaki et al., 1989). However, growth inhibitory effects of TGF-ß on SFME cells are small and variable, even though TGF-ß has a profound differentiating effect on these cells. We could not reproduce the inhibitory effect of serum with either TGF-ß or other peptide factors including interferons and interleukines. Still, the recent finding of peptide growth inhibitors such as TGF-ß and others (Miyazaki et al., 1989) encouraged speculations that such factor(s) may be responsible for the growth inhibition we see with SFME cells in the presence of serum or plasma.

Because serum lipoproteins are potentially growth inhibitory (Ito et al.,1982) we used delipidated human plasma as starting material to study the growth inhibitory activity of plasma. We find evidence that a peptide factor may be responsible for the inhibition.

MATERIALS AND METHODS

Materials. Powdered medium formulations and antibiotics were obtained from Grand Island Biological Company (Grand Island, NY); sodium selenite and ethylenediaminetetraacetate (EDTA) was from Fisher Scientific (Pittsbugh, PA); sodium bicarbonate powder ('Baker Analyzed' reagent) was from J.T.Baker Inc., (Phillipsburg, NJ); 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid (HEPES) was from Research Organics, Inc. (Cleveland, OH). Gelatin-sepharose was obtained from Pharmacia Chemicals (Piscataway, NJ). Bovine insulin, human transferrin, trypsin, soybean trypsin inhibitor were from Sigma Chemical Corp. (St.Louis, MO). Mouse EGF was obtained from Upstate Biotechnologies (Lake Placid, NY). Plasticware for cell culture was from Falcon. Membrane tubing (Spectra/Por) was from Spectrum medical industries, Inc. (Los Angeles, CA). Concanavalin A-agarose was from Bethesda Research Laboratories, Inc. (MD) and Sephadex G-50 was from Sigma Chemical Corp. (St.Louis, MO). Water for reagents was passaged through a Milli-Q (Millipore) water purification system immediately prior to use.

Cell culture. Culture of Balb SFME cells was essentially as described in detail in Loo et al., (1989a and b). Derivation and characterization of oncogene transformed Balb SFME cells was described in Shirahata et al., (1990); the Ha-ras-myc cl.1 SFME cell line was derived by cotransfecting pSVc-myc and pUCEJ6.6, which contains the human Ha-ras gene (Chang, 1987). SFME cells were grown in a basal nutrient formulation of a 1:1 mixture of Dulbecco-modified Eagle's medium containing 4.5 g/L glucose and Ham's F 12 (F 12:DME medium), supplemented with 1.2 g/l sodium bicarbonate, 15 mM HEPES (pH 7.4), 200 U /ml penicillin, 200 μg/ml streptomycin, 25 μg/ml ampicillin, mouse EGF (50 ng/ml), bovine insulin (10 μg/ml), human transferrin (10 μg/ml), human HDL (10 μg/ml), and sodium selenite (10 nM) on 75 cm^2 surface tissue culture flasks precoated with bovine or human fibronectin (10 μg/ml).

Cells were grown in a humified 5% CO_2-95% air atmosphere at 37°C. Trypsinization and plating in serum-free culture was accomplished by short exposure at room temperature to 0.25 % crude trypsin in 1 mM EDTA/PBS without calcium or magnesium followed by dilution of the cells into soybean trypsin inhibitor

solution (1 mg/ml in F12:DME medium). Cells were then centrifuged, resuspended into F12:DME, and counted. The appropriate cell number was plated in 1 ml of prewarmed medium in culture vessels previously precoated with fibronectin and preincubated in the incubator (5% CO_2-95% air atmosphere at 37°C) for approximately 15 minutes with the remaining portion of medium (final volume 2 ml). Insulin, transferrin, EGF, and HDL were added directly from concentrated stocks into the culture medium immediately after plating the cells.

Insulin stock was 1 mg/ml in 20 mM HCl and transferrin stock was 2.5 mg/ml in F12:DME medium; both were filter sterilized. EGF was reconstituted in PBS (50 μg/ml). Fibronectin stocks (about 1mg/ml in 50 mM Tris pH 7.5, 4 M urea) were filter sterilized.
Stocks of insulin, transferrin, EGF, and fibronectin were stored at -86°C in aliquots until needed, then stored at 4°C after thawing. HDL was prepared for cell culture as described in Loo et al.,(1989). Fibronectin was prepared by gelatin-affinity chromatography (Rouslathi et al., 1982, Loo et al.,1989).

Growth inhibition bioassay. Cells were detached from the stock flask by trypsinization. Trypsin was

inactivated by addition of trypsin inhibitor solution followed by fresh nutrient medium. SFME cells were centrifuged, resuspended, counted and plated into a final volume of 2 ml of nutrient medium, and supplemented with EGF, insulin, transferrin, HDL, sodium selenite, bicarbonate and antibiotics as described above, with plasma, serum or column fractions as indicated. The untransformed SFME cells were plated at lower density ($0.5x10^5$/35mm dish) than the transformed SFME ($1x10^5$/35 mm dish), because they grew more vigorously. In some experiments 250 ng/ml oleic acid was added, because it improved the growth of the SFME cells. Cell number was determined after 5 days of incubation, or otherwise at the indicated times after plating, by counting suspensions of trypsinized cells using a Coulter particle counter. Average variation of single determinations from the mean was less than eleven percent.

Dialysis of plasma and plasma fractions. Membrane tubing (3500 M.W. cutoff) was boiled for about ten minutes in 1 mM EDTA, 2% bicarbonate solution and rinsed with Milli-Q water. Tubes were stored in boiled water at 4°C. Dialysis was performed at 4°C with stirring.

Dialysis conditions for the growth inhibitory activity in human delipidated plasma were established as follows: 1.5 ml aliquots of delipidated human plasma that contained the KBr from the delipidation process were dialyzed against 1.5 liter of 10 mM HEPES, pH 7.0, 0.15 M NaCL, 0.1 mM EDTA. 2 ml of human plasma that were the source of the delipidated plasma was also dialyzed against 2 liters of 10 mM HEPES, pH 7.0, 0.1 mM EDTA, 0.15 M NaCl. Dialysis occured over a period of 24 hours with three changes of buffer after 6 hours of dialysis. The samples were sterile filtered (2 μm) and tested on Balb SFME and Ha-ras-myc Cl.1 Balb SFME in 200, 100, 50, and 25 mircoliter doses. Cell number was determined after 5 days.

Preparation of delipidated human plasma. Delipidated human plasma was prepared by KBr ultracentrifugation of freshly drawn citrated human plasma after platelets were removed by centrifugation. The procedure for HDL preparation is described in Loo et al. (1989). The lower phase of the last centrifugation, containing the delipidated plasma, was pooled and dialyzed extensively against 10 mM HEPES, pH 7.0, 0.15 M NaCl using a 3.5 KD cut-off tubing. (Dialysis was

performed over several days with multiple buffer changes until salt was diluted more than 10^5-fold). The dialyzed, delipidated plasma was then heat-treated (60°C) for 30 minutes to achieve clotting and inactivation of complement. Delipidation using organic solvents was performed according to Rothblat et al., (1976).

Methanol precipitation of human plasma and delipidated human plasma. To delipidated human plasma, at 4°C, was added 1/3 plasma volume of ice-cold methanol. This was mixed quickly and left on ice for 1 hour. The precipitate was centrifuged (4°C, 15 minutes, 30,000 g), the supernatant discarded and the pellet resuspended in 10 mM HEPES, pH 7.0, 0.15 M NaCl (1/3 of the original plasma volume). This was left at 4°C overnight to redissolve. Remaining insoluble material was centrifuged (30,000 g, 15 minutes, room temperature) and the dissolved precipitate was sterile-filtered (0.2 μm).

Concanavalin A-agarose chromatography. A concanavalin A-agarose column (31 cm x 2.5 cm, about 150 ml bed volume) was equilibrated by washing extensively (e.g. 2 l) with running buffer (10 mM HEPES, pH 7.0,

0.15 M NaCl). The sample was applied and the column washed with about 300 ml running buffer at 16 ml/h. Fractions were eluted with 200 ml 1 M α-methyl-D-mannoside in running buffer (20 ml/h). Final wash was to baseline of A_{280} (about 0.1) with runnning buffer. All runs were at 4°C. Storage buffer was 20 mM Tris, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.02 % NaN_3, pH7.4.

Sephadex G-50 chromatography. Eluate fractions from concanavalin A chromatography were pooled and dialyzed extensively against 200 mM acetic acid. The dialysate was vacuum-concentrated about tenfold, redialyzed against 200 mM acetic acid and sterile filtered (0.2 μm). A 2 ml sample was applied to a G-50 Sephadex column (1 cm diameter, 45 cm length, 35 ml bed volume) equilibrated in 200 mM acetic acid. Chromatography was developed with 200 mM acetic acid (5-6 ml/h, 4°C). 1 ml fractions were collected.

RESULTS

Inhibitory activity is not dialysable. Extensive dialysis against 10 mM HEPES, pH 7.0 or pH 8.0, 0.15 M NaCl, 0.1 mM EDTA or DME, pH 7.4, did not remove the growth inhibitory activity from delipidated and nondelipidated human plasma. 50 % inhibition with respect to control (no plasma added) was seen at approximately 2.5 mg/ml protein (see figure 1). Dialysis against 10 mM HEPES, pH 6.0, 0.15 M NaCl, 0.1 mM EDTA reduced the activity slightly (50 % inhibition at 3.5 mg/ml). Dialysis against low salt solutions reduced the activity significantly. For example, after dialysis against buffer containing 0.05 M NaCl, 50 % inhibition occurred at 4.5 mg/ml protein. The same protein concentration caused only a 35% inhibition after dialysis with no salt present in the dialysis buffer. This may indicate that the activity is a protein or is bound to a protein that needs salt for solubilization. Dialysis of freshly drawn citrated human plasma against 10 mM Hepes, pH 7.0, 0.15 M NaCl, 0.1 mM EDTA reduced the inhibitory activity, but a significant amount remained. The procedure possibly removed dialyzable

inhibitors like triiodothyronine or hydrocortisone. Delipidated human plasma dialyzed against 10 mM HEPES, pH 7.0, 0.15 M NaCl, 0.1 mM EDTA was of the same inhibitory activity as undialyzed undelipidated plasma. Delipidation may remove growth promoting activities including some growth-stimulatory lipoproteins. Most activity was lost through reduction or omission of the salt content of the buffer. Extensive dialysis of delipidated human plasma against 1 M acetic acid did not remove the inhibitory activity from the plasma. In this property the activity resembles that of many low molecular weight peptide growth factors which are also stable in acidic conditions.

The inhibitory activity precipitates out in low ionic strength; purification using methanol precipitation. Delipidated human plasma containing 10.17 M KBr from the delipidation process was extensively dialyzed against water (100 ml plasma were dialyzed against 4 liter water with 9 changes over 40 hours). The plasma was stored at -20°C before further use. After thawing, the precipitate that formed was removed by centrifugation and the pellet resuspended in 10 mM HEPES, pH 7.0, 0.15 M NaCl, 0.1 mM EDTA. The

resuspended precipitate was tested on SFME and Ha-ras-myc SFME in a 4 day growth assay. The resuspended precipitate was strongly inhibitory for SFME cells but not inhibitory for the transformed cells (data not shown). This suggested that precipitation using an organic solvent, e.g. methanol or ethanol, that lowers the dielectric constant of water might provide a preliminary purification of the inhibitory activity.

We found that 33 % v/v methanol quantitatively precipitated the growth inhibitory activity from nondelipidated and delipidated human plasma. The pellet could be redissolved in HEPES saline (10 mM HEPES, pH 7.0, 0.15 M NaCl) and was enriched 2-3 fold in specific activity with respect to protein concentration (see figure 2). This procedure left all the albumin in the supernatant, as judged by polyacrylamide gel electrophoresis. It is possible that the 33 % v/v methanol liberates some of the otherwise toxic lipids into the supernatant. Delipidation of the human plasma proteins according to the method of Rothblat et al. (1976), which consists of precipitation of the proteins in ten times the plasma volume of acetone-ethanol (1:1 by volume) and a subsequent wash of the precipitate with

diethyl-ether, did not decrease the inhibitory activity of the resuspended plasma proteins (data not shown).

The inhibitory activity in human plasma binds to concanavalin A-agarose and can be eluted with α-methyl-D-mannopyranoside. Concanavalin A is a lectin that binds structures containing alpha-linked mannose, such as in N-acetylglucosaminyl core disaccharide in asparagine-linked glycoproteins. Delipidated human plasma was methanol precipitated, resuspended, filtered (0.2 μm) and chromatographed on concanavalin A-agarose. The chromatogram consisted of three peaks. The fractions with higher A_{280} values in the first peak were turbid. This peak probably represents aggregated material that cannot bind to the matrix. About 50 % of the total input protein was in the second peak, which contained material that was retarded by the column. This peak did not contain any measurable inhibitory activity (figure 3) and consisted mainly of gamma-globulin (judged by SDS polyacrylamide gel electrophoresis). Some inhibitory activity was found in the first peak, but the bulk of activity was eluted as a third peak with α-methyl-D-mannoside. (First and second peak fractions were assayed directly after

sterile filtration (2 μm), eluate peak fractions were dialyzed extensively against 10 mM HEPES, pH 7.0, 0.15 M NaCl, sterile filtered and assayed). The first peak and the third (eluate) peak were of about the same specific inhibitory activity (figure 4). The purification of activity was approximately 1.5-2 fold with respect to protein concentration of the methanol precipitate of delipidated human plasma (figure 4). The material of the first peak and the eluate peak was also inhibitory to the transformed cells, but a distinct differential remained between effects on the normal and transformed cells.

Further purification of the inhibitory activity from concanavalin A-agarose chromatography by Sephadex G-50 chromatography. Eluate fractions from concanavalin A-agarose chromatography of the 33% v/v methanol precipitate of human delipidated plasma were dialyzed against 200 mM acetic acid and vacuum concentrated to A_{280} of 40. 2 ml was run on a Sephadex G-50 column (1x45 cm) in 200 mM acetic acid. Fractions (1ml) were pooled as indicated (figure 5), because the inhibitory activity was expected to be spread over several fractions. The pools were chosen so they would establish, if the

activity was excluded by the gel (molecular weight>circa 30,000) most proteins in the concanavalin A-agarose eluate as judged by gel electrophoresis), or retained by it. Pools were vacuum concentrated and assayed on untransformed SFME and Ha-ras-myc cl.1 SFME cells. The activity was present in the retarded portion of the chromatogram, whereas the majority of proteins were in the void volume. When the Sephadex G-50 column was developed in 10 mM HEPES, pH 7.0, 0.15 M NaCl, instead of acetic acid, the inhibitory activity chromatographed with the excluded material (data not shown). In another experiment eluate fractions of the concanavalin A chromatography were pooled and chromatographed on the Sephadex G-50 column in 200 mM acetic acid. Fractions that followed the peak of unretarded material (where the inhibitory activity was to be expected, about 10 fractions) of three column runs were collected, vacuum concentrated, dialyzed against 50 mM acetic acid and assayed in a bioassay. This G-50 preparation was strongly inhibitory to Balb SFME cells with 50 % inhibition requiring about 20 μg/ml protein. Low doses of acetic acid (<2.5 mM) in the culture medium were not inhibitory. It was also inhibitory to the transformed

cell line with 50 % inhibition at about 40 µg/ml. Thus, the differential effect was essentially lost in this purification. It cannot be excluded that the inhibitory activity in human plasma changes its behavior during its purification, because other, interacting substances have been lost (see 'DISCUSSION' below). Still, the activity, like the activity in unfractionated human plasma, was largely susceptible to inactivation by heating to 100 °C for three minutes (see figure 6). Inactivation by boiling could be explained by loss of tertiary structure of a protein, however, we could not demonstrate that the activity is destroyed by digestion with proteases (data not shown). Further, it was not inactivated by reduction with 50 mM dithiothreitol at pH 8.0, a condition that would inactivate TGF-ß (Assoian et al., 1983). The untransformed SFME cells are normally rather elongated cells, but became very small and rounded in the presence of the G-50 preparation, and stuck tightly to the culture dish. The cells exhibited this striking morphology even at lower doses, where the preparation did not inhibit the cells from several rounds of doubling.

It should be noted that small amounts of human

plasma and all preparations described here caused some growth stimulation of SFME cells. Microscopic examination of the cells suggested that this may be because of better attachment of the cells.

DISCUSSION

The use of serum-free cell culture to study growth inhibiting activities in plasma/serum. Serum-free cell culture opens the possibility to study growth inhibitory activities present in blood plasma or serum that may otherwise not be detectable. Cell lines that have been derived in serum-containing media often lose their responsiveness to these factors. The use of a serum-free derived cell line circumvents this problem. However, serum-free cell culture can also pose problems in the study of growth inhibitory activities, since the cells are more sensitive to perturbing substances than cells growing in serum-containing media. Especially fatty acids and other lipids may harm the cells if not buffered by proteins such as albumin. The use of delipidated plasma or serum for fractionation procedures may eliminate possible unspecific growth inhibition by lipoproteins and/or lipids that are released. Since plasma and serum also contain growth promoting activities that can overshadow the inhibiting activities it is important to provide the most optimal growth conditions for the cells in a bioassay of the growth

inhibiting activities. One should try to maximize the mitotic potential of the cells by providing all supportive activities plasma or serum may offer. In our case beneficial components included attachment factors and some lipids, and were provided by fibronectin and 250 ng/ml oleic acid.

Nature of the growth inhibiting activity. We achieved a 50 to 100-fold purification of the growth inhibiting activity from delipidated human plasma. In the course of this purification the activity behaved like a macromolecular entity (molecular weight>3.5 KD). It was not dialysable, even against 1 M acetic acid, which disrupts binding of many ligands to binding proteins. It could be precipitated by 33% v/v methanol, which precipitates most plasma proteins with the exception of albumin. It bound to concanavalin A-agarose, a lectin matrix, and it therefore must be either glycosylated or else bound very tightly to a glycosylated entity. The activity that was retained by Sephadex G-50, was not dialysable in 200 mM acetic acid and was inactivated by boiling for three minutes.

In the course of the purification the differential

growth inhibitory effect on untransformed versus transformed SFME cells disappeared, and in the final preparation there was little difference in activity on normal and transformed cells. It is possible that our fractionation procedures removed factors that allow the transformed cells to overcome the inhibitory activity, while the untransformed cells are not responsive to these substances. Another explanation would be that several growth inhibitory activities are present in the plasma, one or several with a differential effect and others without a differential effect. We may have purified an activity without the differential effect. This possibility must be considered, since the yield of activity in our procedure is low (4% total recovery of inhibitory activity).

The final preparation did not induce the expression of GFAP, as does TGF-ß, nor did it prevent cell death in absence of EGF (data not shown). Although TGF-ß causes expression of astrocyte markers in SFME cells, it does not prevent exponential growth of these cells. Since astrocytes are obviously under tight growth control <u>in vivo</u>, a mechanism must exist that exerts this control. An astrocyte-specific mitogen

inhibitor has been identified (Nieto-Sampedro ,1988) in brain extracts that is related to the EGF receptor. Interestingly, a liver extract also containing EGF receptor crossreactivity was also growth inhibitory to astrocytes. We observed an activity in conditioned serum-free medium of the human liver cell line HepG2 that had a growth inhibitory effect on the untransformed SFME cells but not on transformed SFME cells (data not shown). The relationship of the inhibitor reported by Nieto-Sampedro and the activity in the HepG2 conditioned medium or in plasma we studied is not known.

Figure 1: Bioassay of human plasma inhibitory activity after dialysis. Counts of Balb SFME cells were carried out after 5 days. Cells were plated at 10^5 cells/35 mm dish.

triangle: undialyzed human plasma in culture medium.

circle: human plasma, dialyzed against 10 mM HEPES, pH 7.0, 0.15 M NaCl, 0.1 mM EDTA in culture medium.

square: delipidated human plasma, dialyzed against 10 mM HEPES pH 7.0, 0.05 M NaCl, 0.1 in culture medium.

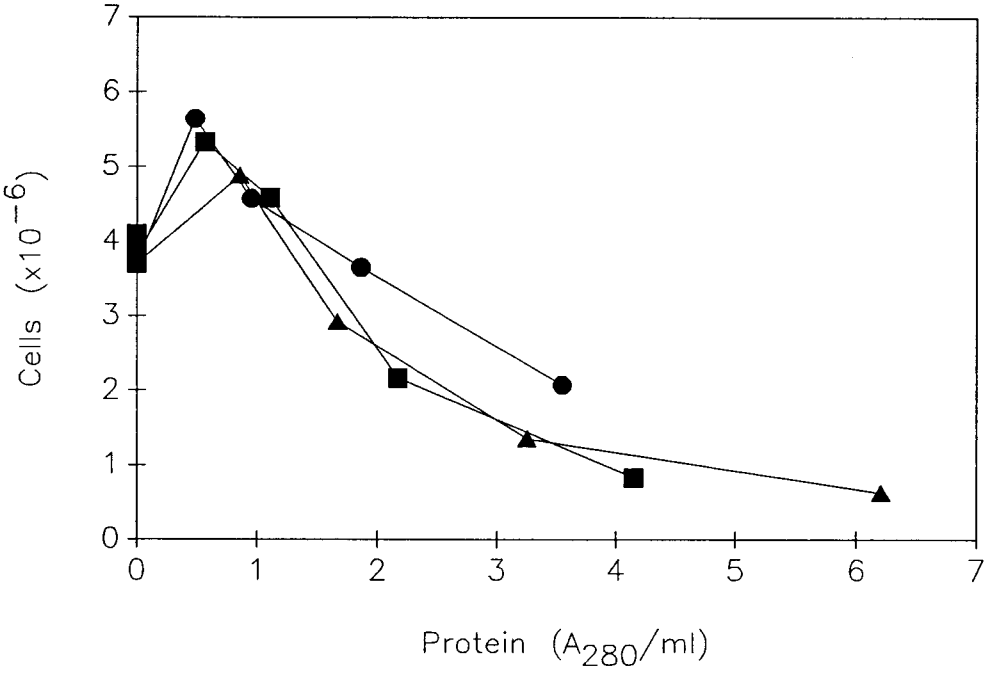

Figure 1

Figure 2: Bioassay of methanol precipitate of delipidated human plasma. Balb SFME cells were plated at $0.5x10^6$, Ha-*ras*-*myc* clone-1 SFME were plated at $1x10^6$ cells\35 mm dish. Cell count was after 5 days.

squares: delipidated human plasma on Balb SFME cells.

circles: resuspended methanol precipitate on Balb SFME cells.

triangle: resuspended methanol precipitate on Ha-*ras*-*myc*-clone 1 cells

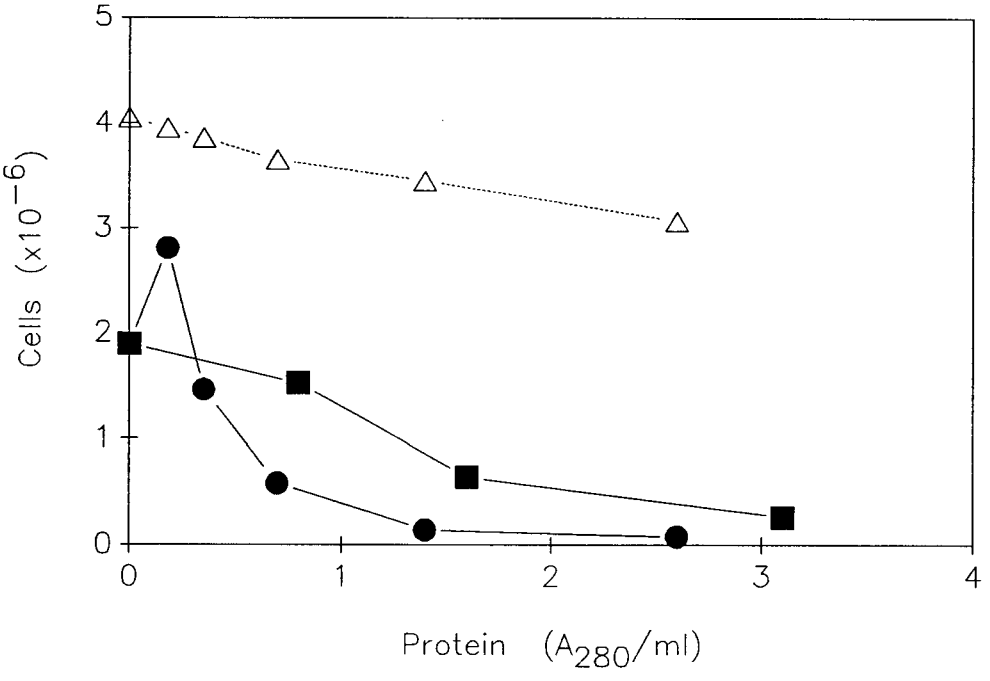

Figure 2

Figure 3: Bioassay of inhibitory activity after concanavalin A-agarose chromatography of human plasma inhibitory activity in 33 % v/v methanol precipitate. Balb SFME cell number was determined after 4 days of growth.

circle: peak I

triangle: peak II

square: peak III (α-methyl-D-mannoside eluate)

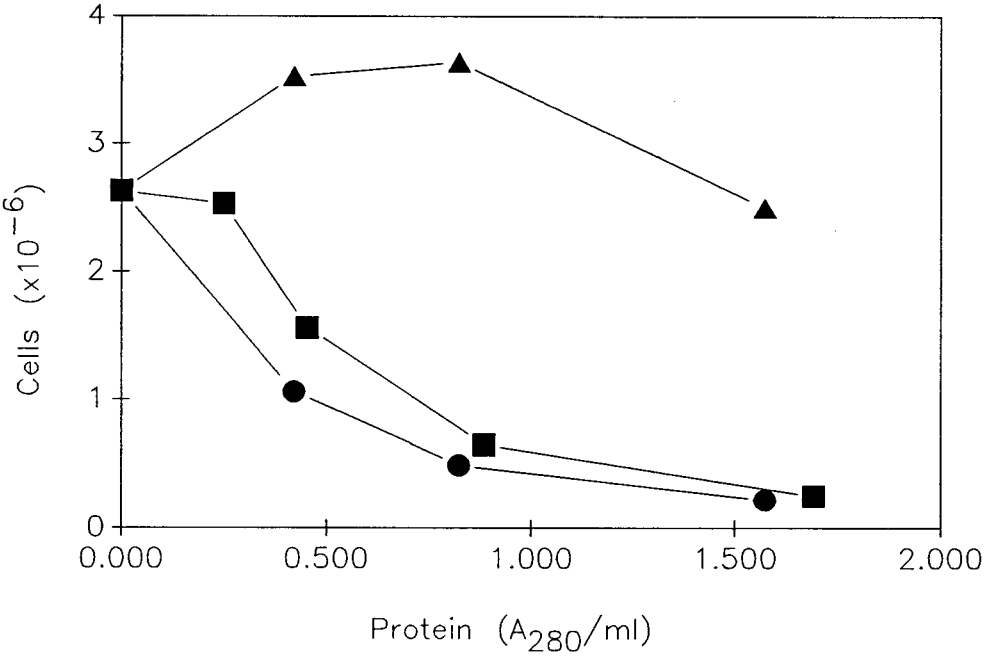

Figure 3

Figure 4: Bioassay of inhibitory activity of concanavalin A-agarose eluate. Balb SFME cells were plated at $0.5x10^6$ cells/35 mm dish, the Ha-ras-myc transformed cells at $1x10^6$cells/35 mm dish. Cells were counted after 5 days of growth.

square: resuspended methanol precipitate of delipidated human plasma on untransformed cells

circle: dialyzed peak fraction of eluate on untransformed cells

triangle: dialyzed peak fraction of eluate on transformed cells

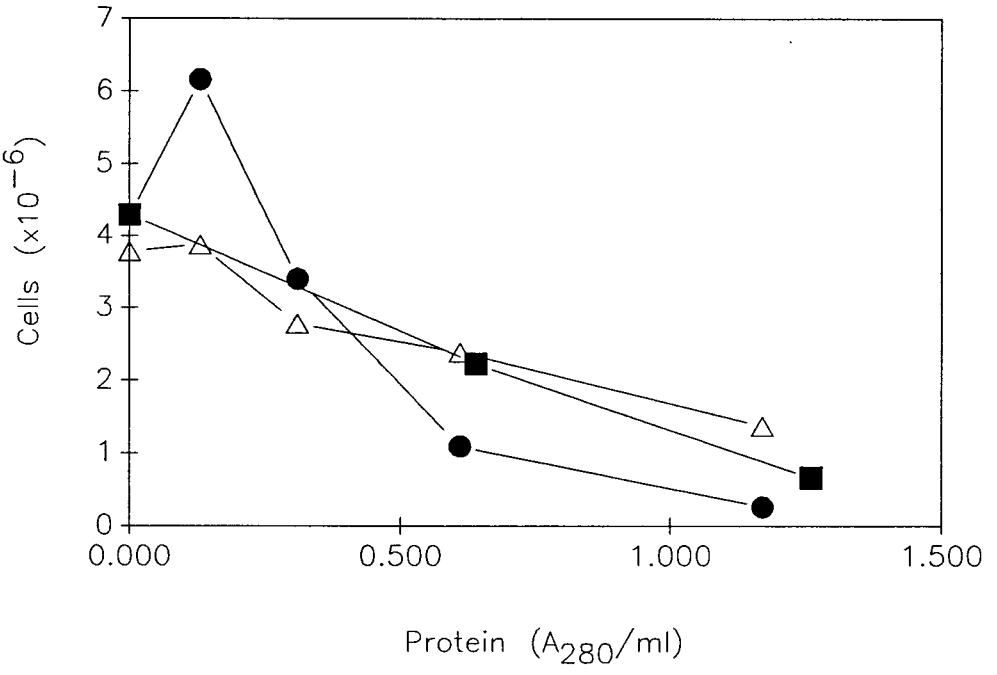

Figure 4

Figure 5: Molecular-sieve chromatography of growth inhibitor fraction. Concanavalin A eluate of 33 % methanol precipitate of human delipidated plasma was dialyzed against 200 mM acetic acid, vacuum concentrated and chromatographed on a 1x45 cm-column of Sephadex G-50 in 200 mM acetic acid.

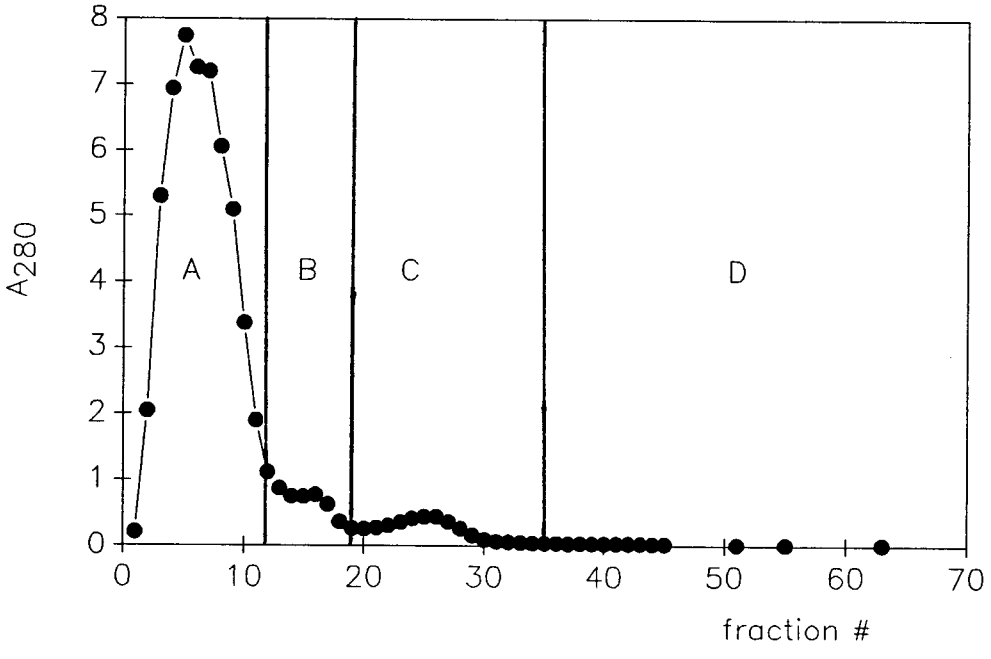

Figure 5

Figure 6: Dose-response curves of the growth inhibitory activity after purification by Sephadex G-50 gel chromatography in 200 mM acetic acid. Balb SFME cells were plated at $0.5x10^6$/35 mm dish, Ha-ras-myc clone-1 SFME cells were plated at $1x10^6$/35 mm dish.
Fractions were in 50 mM acetic acid. Identical volumes of 50 mM acetic acid were added to control platès. A portion of the preparation was boiled for three minutes and then tested;
full circles: BalbSFME/G-50 prep
full square: BalbSFME/boiled G-50 prep
empty triangle: Ha-ras-myc clone-1 SFME/G-50 prep

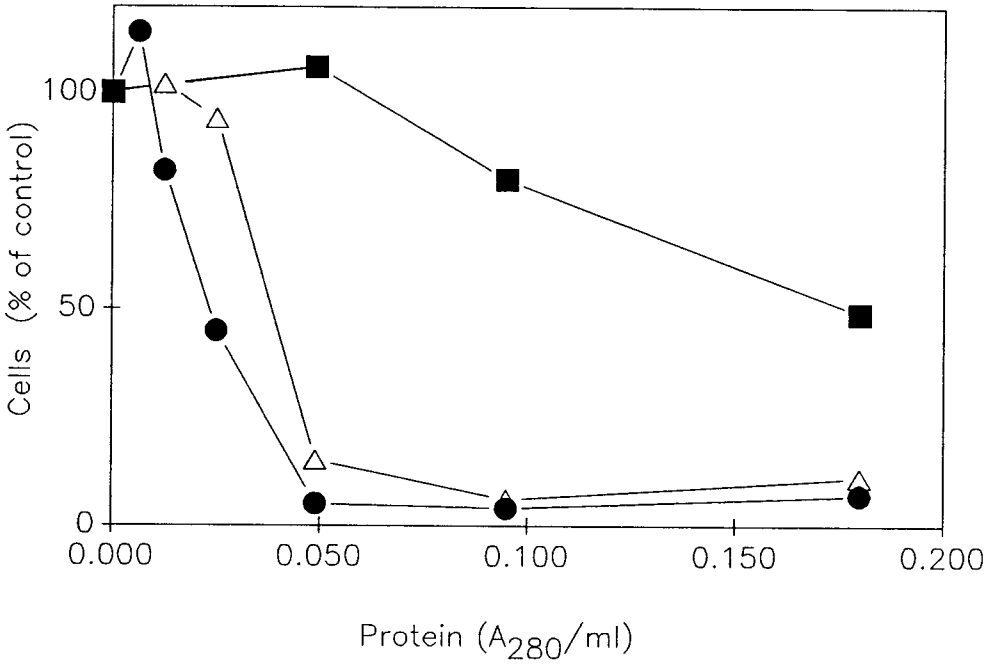
100
50
0
Cells (% of control)
0.000
0.050
0.100
0.150
0.200
Protein (A_{280}/ml)

Figure 6

Table 1: Bioassay of fraction pools of Sephadex G-50 chromatogram. Fraction pools are as indicated in figure 5. Balb SFME cells were plated at $0.5x10^5$, Ha-*ras*-*myc* cl.1 SFME cells at $1x10^5$. Counts were after five days.

TABLE 1:

Bioassay of fraction pools of Sephadex G-50 chromatogram.

	Balb SFME		Ha-*ras*-*myc* cl.1	
	Cell number (% of control)	Protein (A_{280}/ml)	Cell number (% of control)	Protein (A_{280}/ml)
pool A	130	0.27	79	0.27
pool B	15	0.2	50	0.2
pool C	57	0.05	43	0.05
pool D	33	0.04	42	0.04

Chapter III

IDENTIFICATION OF A TGF-ß REGULATED, BRAIN-SPECIFIC mRNA IN SFME CELLS

SUMMARY

Using the technique of differential screening of a cDNA library a calf serum- and TGF-ß- regulated mRNA species was identified in SFME cells. This RNA is brain-specific and approximately 8.5 kilobases long. Picomolar quantities of TGF-ß caused an increase of this message in SFME cells within four hours. This increase was reversed when TGF-ß is removed from the culture medium.

INTRODUCTION

Serum-free mouse embryo (SFME) cells are derived in medium in which serum is replaced with growth factors and other supplements. They display unusual properties. (i) They do not lose proliferative potential or show

gross chromosomal aberration upon extended culture, (ii) they depend on epidermal growth factor for survival , and (iii) are reversibly growth inhibited by plasma and serum (Loo et al., 1987; Loo et al.,1989 a; Loo et al., 1989 b; Loo et al.,1990).

Treatment of SFME cells with serum or transforming growth factor beta (TGF-ß) causes the appearance of glial fibrillary acidic protein (GFAP) and GFAP mRNA (Sakai et al.,1990). GFAP is a specific marker for astrocytes (Lazarides, 1982; De Vellis et al., 1986; Morrison et al., 1985). In addition to GFAP, TGF-ß regulates the protease inhibitor cystatin C in SFME cells (Solem et al.,1990).

Transforming growth factor beta, a serum component (Bjornson et al., 1982; Masui et al., 1986) is a peptide that influences differentiation of various cell types in vitro and in vivo, particulary those of mesodermal origin. However, reports on effects of TGF-ß on cells of neuroectodermal origin are scarce. This is also true for cells of the astrocytic lineage. TGF-ß1 and TGF-ß2 have been shown to suppress rat astrocyte autoantigen presentation and antagonize hyperinduction of class II major histocompatibilty complex antigen expression by

interferon-gamma and tumor necrosis factor-alpha (Schluesener et al., 1990). Robertson et al. (1988) found that TGF-ß stimulated phosphoinositol metabolism and translocation of protein kinase C in cultured astrocytes. Saad et al. (1991) observed an effect of TGF-ß2 on the regulation of neural recognition molecule expression by cultured astrocytes. The finding of our laboratory that TGF-ß induces GFAP in SFME cells suggests that TGF-ß may play a role in astrocyte development in vivo. TGF-ß may contribute to the phenomenon of gliosis, the hypertrophy and increase in GFAP in astrocytes in response to injury or chronic disorders of the brain. Recently, TGF-ß has been identified by immunochemistry in the brain tissues of patients with aquired immune deficiency (but not in control brain). The TGF-ß staining was localized to astrocytes (as well as cells of monocytic lineage), especially in areas of brain pathology (Wahl et al., 1991). These results stress the importance of further investigating the action of TGF-ß on cells of the astrocytic lineage. It is interesting to investigate whether other brain specific messages are induced by TGF-ß in SFME cells. Such a message may serve as

additional marker of differentiation. It may provide further insights into brain specific action of TGF-ß.

We used the technique of differential screening of a cDNA library to identify a novel, large mRNA species that is reversibly induced by serum and TGF-ß in SFME cells and that is brain specific.

MATERIALS AND METHODS

Cell culture and RNA isolation. Procedures for culture of Balb/c SFME cells have been described in detail in Loo et al. (1987), Loo et al. (1989 a), Loo et al. (1989 b). RNA isolation was done as follows. Cells growing in plastic dishes were washed twice with ice-cold PBS. They were then gently scraped off the dish (in 1-2 ml of PBS) with a rubber policeman and pelleted. Cells were resuspended in 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, 200 μg/ml heparin (Sigma Chemical Company, MO) and lysed by adding 0.5 % Nonidet P-40 (Sigma Chemical Company, MO). After brief mixing the lysate was centrifuged at 4000xg for 10 min. 1% SDS and 10 mM EDTA was added to the supernatant followed by extraction twice each with phenol, phenol/chloroform (1:1), and chloroform/isoamylalcohol (24:1), and ethanol precipitation. Residual DNA was removed with RNAse free DNAse I in the presence of vanadyl riboside complex (both from Bethesda Research Laboratories, MD). Poly A-containing RNA was isolated as described by Davis et al. (1986). Mouse tissue mRNA was obtained from Clonetech Inc., (CA).

Northern blot analysis. RNA (20 μg/lane) was fractionated by electrophoresis on formaldehyde/1.2 % agarose gels and transferred overnight to nitrocellulose membranes (0.45 μm pore size, Schleicher & Schuell, N.H.), as described by Maniatis et al. (1982). DNA probes were ^{32}P-labeled by random primer extension using a kit from Boehringer Mannheim, α-^{32}P-radiolabeled nucleotides (3000 Ci/mmol) were from NEN Research Products, (DE). Membranes were prehybridized for 4 hr and then hybridized with probe (about 10^6 cpm/ml) overnight in 10 % dextran sulfate (Pharmacia, N.J.), 40 % formamide, 4x SSC, 10 x Denhardt's solution, 0.2 M Tris-Cl, pH 7.4, 200 μg/ml boiled sonicated salmon sperm DNA at 42°C (G+C content < 50%) or 57°C (G+C content>50%). Filters were washed three times in 2 x SSC, 0.1 % SDS at room temperature for 15 minutes, then twice in 0.1 x SSC, 0.1 % SDS at 65°C for 15 minutes. Autoradiography was carried out on Kodak X-Omat AR film from overnight to several days at -80°C.

Southern blot analysis. A Southern blot with restriction enzyme digested mouse genomic DNA was performed using mouse Genoblot from Clonetech Inc., (CA) according to the manufacturers directions.

Differential screening. A cDNA library was prepared from mRNA of SFME cells, grown in serum-free medium supplemented additionally with 10 % CS for 24 h, using a cDNA synthesis kit (Pharmacia, N.J.). First-strand cDNA synthesis was preformed with Moloney murine leukemia virus reverse transcriptase and oligo $d(T)_{12-18}$ as primer. Second-strand synthesis involved a modification of the procedure of Gubler and Hoffman (1983). To the blunt-ended cDNA an EcoRI/NotI adaptor was ligated to each end. The cDNA was ligated to lambda gt11 arms, packaged and plated using E. Coli strain Y1090 as host. The library contained over 2.4×10^6 unique clones (average size smaller than 200 bp; maximal size 3 kb). Radiolabeled, single-stranded cDNA probes were synthesized using as template mRNA from SFME cells cultured with or without 10 % CS for 24 h. Specific activities were $1\text{-}5 \times 10^7$ cpm/μg mRNA for both probes. Differential screening of phage plaques transferred to duplicate nitrocellulose filters was performed using about 5×10^5 cpm/ml of the radiolabeled probes in hybridization buffer containing 10 % dextran sulfate, 40 % formamide, 4 x SSC, 10 x Denhardt's solution, 0.2 M TrisCl, pH 7.4, 10 μg/ml poly (A) (Pharmacia), and 200

μg/ml sonicated salmon sperm DNA. Hybridization was at 42°C for three days. The filters were then washed three times for 15 minutes with 2 X SSC, 0.1 % SDS at room temperature and twice for 15 minutes with 0.2 x SSC, 0.1 % SDS at 52°C, Filters were exposed to x-ray film up to three days at -80°C with intensifying screen. Plaques that were detected by the CS-induced cDNA probe and not by the cDNA probe derived from SFME cells cultured serum-free were selected and rescreened four times. The cDNA insert from one positive clone was subcloned in pUC18.

Cloning of overlapping cDNA inserts. cDNA inserts labeled with ^{32}P using the random primer method were used to screen various libraries for overlapping cDNAs:
(i) An oligo-(dT)-primed library in lambda gt11 prepared from mRNA of TGF-ß treated SFME cells (Solem et al., 1990, TGF-ß was TGF-ß1 from porcine platelets, R&D Systems, Inc., MN), (ii) an oligo-(dT) primed library in lambda gt10 prepared from mouse brain mRNA (from ATCC, no. 37431), and (iii) a random primed mouse brain cDNA library (Cat.# ML1024a, lot# 2411, Clonetech, CA) in gt10. Filters were hybridized as described for the differential screen, with the exception that

hybridization was only overnight with 25-100x10^3 cpm/ml probe. Filters were washed three times for 15 minutes with 2 x SSC, 0.1 % SDS at room temperature, then two times with 0.1 x SSC, 0.1 % SDS at 52°C.

Sequence analysis. Sequencing of the cDNA inserts in pUC18 was performed by the Oregon State University Center for Gene research using an automated laser sequencer (ABI systems,CA). Sequence data bank searches were done with the FastDB program of IntelliGenetics.

Stability of TGF-ß regulated mRNAs in the presence of α-amanatin. SFME cells were grown 24 hours in the presence of TGF-ß, the medium was then changed to medium without TGF-ß, and α-amanatin (1 μg/ml, from Sigma Chemical Corp., MO) was added. TGF-ß was again added 15 minutes after α-amanatin addition to half of the plates. After 0, 3, 6, 8 hours of incubation RNA was isolated, and Northern hybridization blot analysis carried out. Probes were a cDNA piece that hybridized to an approximately 8.5 kb, TGF-ß regulated mRNA (ß8), a ß-actin cDNA probe (gift from Dr. J. Pipas, University of Pittsburgh), and a cystatin C cDNA probe (Solem et al., 1990).

RESULTS

Identification of a CS/TGF-ß regulated RNA in SFME cells. Using the technique of differential hybridization, we identified a RNA that is regulated by calf serum or TGF-ß in SFME cells. Poly A-containing RNA of SFME cells that were cultured 24 hours in the presence of 10 % calf serum was purified to construct a cDNA library in gt11. This library was screened by dublicate filters with radiolabeled cDNA prepared from poly A-containing RNA of CS or serum-free treated SFME cells. Plaques that were labeled by the 'calf serum'-cDNA but not by the serum-free cDNA were rescreened in four rounds. One final candidate clone contained a 183 bp insert that hybridized to an approximately 8.5 kb RNA that was present in total RNA of calf serum or TGF-ß treated SFME cells, but not in serum-free grown cells. RNA size was estimated using the ribosomal bands as size markers. We named this RNA ß8. When probing total cytoplasmic RNA of SFME cells faint bands of the size of the 18S and 28S RNAs appeared on the Northern blot in addition to the 8.5 kb band. This probably represents hybridization to the ribosomal RNA and degraded ß8 RNA,

since those bands were absent when poly A-containing RNA was probed. It may be that during gel electrophoresis when the formamide of the loading buffer became diluted degraded ß8 RNA bound to ribosomal RNA and comigrated with it. This effect could be minimized by chosing a piece of cDNA for probe that was richer in G+C than the rest of cDNA, allowing hybridization at higher temperature (57°C).

The TGF-ß regulated RNA is brain specific. A Northern blot was performed using poly A-containing RNA of different mouse tissues (brain, heart, kidney, liver, lung, pancreas, skeletal muscle, spleen, and testis). The 183 bp cDNA insert found by the differential screening was used as probe. The 8.5 kb RNA was detected in brain RNA, whereas no band was present in the other tissue RNAs (figure 7). Balb 3T3 and 10 T 1/2 cells grown in the same culture medium as SFME do not express the RNA even in the presence of TGF-ß (figure 8).

Cloning the 3' part of the TGF-ß regulated RNA ß8. A Southern blot of mouse genomic DNA digested with

various restriction enzymes was probed with the 183 bp fragment. For all restriction enzyme digests only one band was visible (figure 9). This indicates that the TGF-ß regulated RNA represents a single copy gene.

Overlapping clones were obtained using radiolabeled cDNA fragments as probes screening various cDNA libraries. Cloning was straightforward until a segment piece of B1 mouse repetitive element was reached. From there on library screening gave clones that had only the repetitive element in common and two clones that were obviously of chimeric nature. While their 3' sequences matched to the sequence of the 8.5 kb RNA, their 5' sequences did not hybridize to the 8.5 kb RNA and their 5' sequences were also not identical with each other. The fact that the divergence begins within the B1 element suggests that the element may be the site of some recombinatory event, either during the synthesis of the cDNA or in the vectors. This may not be surprising since B1 elements are so abundant. When the random primed mouse brain cDNA library was screened with cDNA pieces containing B1 repeats approximately 2-5 % of the clones were labeled. Other approaches for additional sequence failed. An attempt to purify ß8 RNA

by hybrid selection (Jagus et al., 1987) to construct a ß8 enriched cDNA library did not succeed. The cDNA which was selected to bind the ß8 RNA leaked from the nylon membrane and ß8 RNA either did not bind specifically or could not be eluted from the membrane. An approach to clone more sequence using PCR techniques (Frohman, 1990) failed because one ß8 specific primer did not prime at the appropriate site. The combined sequence from the overlapping cDNA pieces was compared with sequences of large data banks. This did not reveal any striking homology with known sequences, but pointed to the presence of a B1 repeat, a rodent repetitive element related to the Alu repeat in humans (Jelinek, 1982). In addition to the B1 repetitive element sequence the sequence revealed a canonical polyadenylation signal (Lewin, 1983), a poly-(A)-tail, and a striking poly-(CA) stretch (CA_{19}) (figures 17 and 18). Similar poly-(CA) streches are found in several mRNA species (e.g. 3'-untranslated tail of rat kidney mitochondrial glutaminase mRNA, Shapiro et al., 1991).

The RNA ß8 is specifically induced by TGF-ß in SFME cells. SFME cells were cultured in the presence of

various peptide growth factors (fibroblast growth factor, platelet derived growth factor, nerve growth factor, and transforming growth factor-ß) at 10 ng/ml for 48 h. RNA was then extracted and Northern blot analysis was performed using a cDNA fragment as probe. Only SFME cells treated with TGF-ß expressed ß8 RNA (figure 10). In addition, 3,5,3'-triiodothyronine (T_3) at 10 nM and interferon-gamma at 10 ng/ml did not cause an increase of ß8 RNA (figure 11). A Northern blot of RNA extracted from SFME cells treated with 10 ng/ml TGF-ß for various lengths of time shows that an increase is apparent 4 hr after addition of this factor (figure 12). Addition of TGF-ß for 24 hours and subsequent removal for 24 hours caused a complete reversion of the increase. TGF-ß at 1 ng/ml (40 pM) caused a maximal response in SFME cells (figure 13).

Comparison of the TGF-ß regulation of the ß8 RNA and cystatin C mRNA in SFME cells. The effect of TGF-ß on the stability of the 8.5 kb RNA was investigated in SFME cells. SFME cells were first incubated with TGF-ß for 8 hour to increase the starting level of the message. Then the cells were incubated with α-amanatin

(1 μg/ml) to block further transcription (Drew et al., 1989). The rate of depletion of the RNA was then determined by Northern blot hybridization analysis of cells incubated with or without TGF-ß (10 ng/ml) in the culture medium. That rate was then compared to the rate of depletion of cystatin C mRNA under the same conditions. After 8 hours of incubation with α-amanatin and TGF-ß there was a depletion of cystatin C mRNA without TGF-ß, but not with TGF-ß (Solem et al., 1991, unpublished data). However, there was no differential in RNA stability with the 8.5 kb RNA (figure 14). In contrast to cystatin C, the increase of level of ß8 RNA in SFME was adversely affected by 1 μg/ml cycloheximide in the culture medium (figure 15).

The regulation of ß8 in some transfected Balb SFME cell lines was investigated. Derivations and characteristcs of these lines are described in Shirahata et al. (1990). Ha-ras Balb SFME cells and neu Balb SFME cells were transformed by the human Ha-ras oncogene and the rat neu oncogene respectively. Selection was growth in the absence of EGF. Neo SFME were transfected with a plasmid (pSVneo) containing the bacterial aminoglycoside phosphotransferase gene under control of

the SV40 early promoter (Southern and Berg, 1982) and selected in G418. Neo-neu SFME were derived by cotransfection of a plasmid containing the rat neu oncogene under the control of the SV40 promoter (pSV2neuNT) (Bargmann et al., 1986 a,b) and the pSVneo plasmid. Selection was in G418.

Interestingly, while Ha-ras-transformed SFME cells expressed GFAP even in the absence of serum or TGF-ß (Sakai et al., unpublished data), they did not do so with ß8 RNA. Surprisingly, two SFME cell lines transfected with the neomycin resistance marker pSVneo expressed ß8 even in the absence of serum or TGF-ß (figure 16).

DISCUSSION

We identified a large, brain-specific RNA that is regulated by TGF-ß and CS in SFME cells. Increase in RNA level was observed within four hours after treatment and was reversible upon removal of TGF-ß. Picomolar concentrations of TGF-ß in the range of the K_d of the known TGF-ß receptors (Massaguè et al., 1990) were sufficient to cause a significant increase. We named this RNA ß8. No other specific factor investigated caused an increase of ß8 in SFME cells. The RNA was present in poly A-containing RNA, it contains a polyadenylation signal and a poly-(A)-tail. It most likely represents a messenger RNA molecule. Since the combined sequence of the overlapping clones does not reveal an open reading frame this sequence probably belongs to the 3'-untranslated tail of the message. The fact that the TGF-ß induced message is relatively large and has a large 3'-untranslated tail is consistent with the idea that it is a brain-specific message. Brain-specific mRNAs are on average considerably larger than mRNAs not specifically expressed in the brain and also have large 3'-untranslated tails (Sutcliffe, 1988).

Milner and Sutcliffe (1983) estimated from a randomly collected set of rat brain mRNAs that the average length of brain-specific molecules was around 4000 nucleotides, and for the rarer class 5000 nucleotides, whereas the average length of a nonspecific message was around 2000 nucleotides. Brain-specific mRNAs are very large because they encode large proteins and have large 3'-untranslated tails. Examples for such large brain-specific messages are those of the microtubule associated proteins (MAP1, MAP2, tau), the neurofilaments, sodium channels I and II, and synapsin 2 (Milner and Sutcliffe,1983). ß8 RNA is of almost the same size as reported for MAP2a and MAP2b mRNA which is also expressed in cultured astrocytes and reactive astrocytes (Couchie et al., 1985; Charrière et al., 1991; Geisert et al., 1990). In a Northern blot of SFME RNA probed with an endlabeled synthetic oligonucleotide representing nucleotides 5716-5775 of MAP2 (Wang et al., 1988) only the 6 kbp form of MAP2 (MAP2c, Couchie et al., 1985) was detectable, both in the absence and presence of TGF-ß (data not shown).

Another notable feature of the ß8 message is the presence of a B1 repetitive element as illustrated

below. The 46 most 5' nulceotides of ß8 that have been cloned (upper line) are compared with sequence derived from a B1 consensus sequence (lower line, Bains et al., 1989).

```
   CTGGGATTAA AGGTGTGCGC CACCACGCCC GGCTCTACAT TTTTC..
   |||||||||| ||||||| || |||||||||| ||| || |  ||
...CTGGGATTAA AGGCGTGTGC CACCACGCCC AGCTATATTT CTTAT..
```

The B1 repetitive element is 129-bp long (Krayev et al.,1980) and belongs to a family of small moderately repetitive sequences in the mouse genome (approx. 10^5 copies per genome) and is related to the Alu family of dispersed repeats in the human genome. Both of these repetitive DNA families are evolutionary derived from 7SL RNA, the RNA molecule present in the signal recognition particle which mediates the translocation of secretory proteins across the endoplasmatic reticulum (Ullu et al.,1984). These repeats may be mobile elements and are found in mRNA precursor molecules as well as cytoplasmic mRNAs (for review: Jelinek, 1982). Some are also transcribed as discretely sized RNAs by RNA polymerase III. Their function, if any, is unknown.

When transcribed into RNA these repeats may form hairpin structures that were the basis of their initial isolation (Krayev et al., 1980).

Another striking feature of the ß8 sequence is a homocopolymeric repeat consisting of CA doublets. This structure has been identified as the sole unit of another repeated element that is highly conserved throughout eukaryotic genome organization (Hamada et al., 1982). It has been hypothesized that this element has Z-DNA-forming potential in vivo (Hamada et al., 1982) and that these sequences are potential targets for recombination events (Hellman et al., 1988).

In addition to the ß8 RNA, GFAP mRNA and cystatin C mRNA also are known to be regulated by TGF-ß in SFME cells (Sakai et al., 1990; Solem et al., 1990). While GFAP mRNA is brain-specific, cystatin C mRNA is also found in most other tissues (Solem et al., 1990). The time course and dose response of TGF-ß regulation of both ß8 and cystatin C are almost identical. It is especially noteworthy that the ß8 , cystatin C, and GFAP mRNA increase is reversible. In contrast to cystatin C regulation in SFME, ß8 regulation is affected by cycloheximide. TGF-ß does not affect RNA stability of

ß8 in the same way as with cystatin C. This may point to different mechanisms of regulation.

To evaluate the physiological importance of the TGF-ß regulation of mRNAs in cells of the astrocytic lineage, the presence of TGF-ß in the brain has to be investigated. TGF-ß1 immunoreactivity has not been found in the brain tissue of embryonic or adult mice, but in tissue derived from mesenchyme, including the meninges (Heine et al., 1987). By in situ hybridization TGF-ß3 transcript-containing cells have been described in the meninges, choroid plexus and the olfactory bulb, but cells containing TGF-ß1 and TGF-ß2 mRNA were not detectable in other parts of the brain (Wilcox and Derynk, 1988; Pelton et al., 1990). However, an activity in acid/ethanol extract from adult mouse brain that has properties of TGF-ß (Roberts et al., 1981) has been identified. Clearly, the contribution of TGF-ß to brain development and regulation has to be subject of further studies.

Figure 7: Tissue specifity of ß8 RNA. Northern blot of mRNA of different mouse tissues, 2 μg poly A-containing RNA /lane, probed with the 183 bp insert that was first identified in the differential screen (clone A). Poly A-containing RNA from (A) testis, (B) spleen, (C) skeletal muscle, (D) pancreas, (E) lung, (F) liver, (G) kidney, (H) heart, (J) brain.

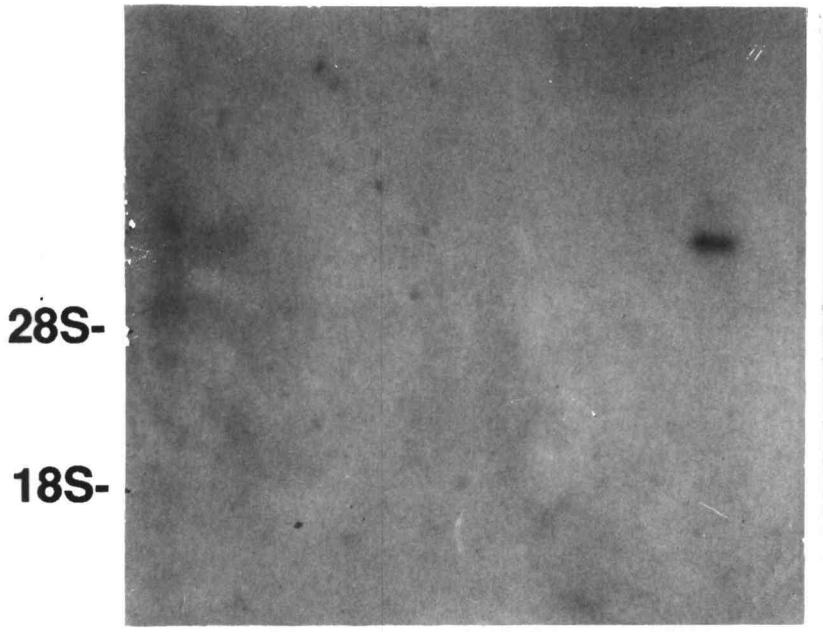

Figure 7

Figure 8: Specific TGF-ß regulation of brain-specific RNA ß8 in SFME cells. Balb SFME cells (A, B), 10T1/2 cells (C, D), and Balb 3T3 cells (E, F) were grown without (A, C, E) and with TGF-ß (B, D, F) for 48 hours in serum-free medium. Cytoplasmic RNA was extracted and Northern blot hybridization analysis was carried out using as probe a 183 bp cDNA clone identified as representing a TGF-ß regulated mRNA (20 μg RNA/lane). The amount of RNA per lane was about equal as judged by the ethidium bromide stained ribosomal RNAs.

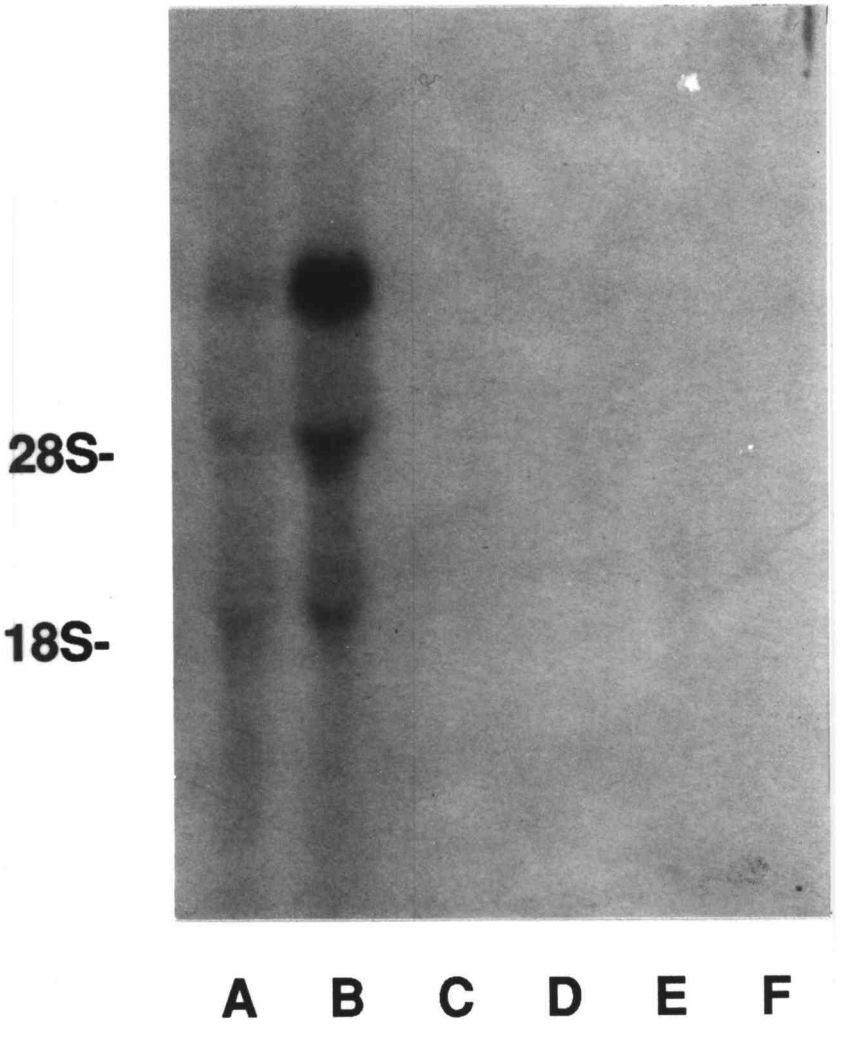

Figure 8

Figure 9: Southern blot of mouse genomic DNA digested with various restriction enzymes. The probe was the 183 bp cDNA piece that was first identified in the differential screen. (A), BglII; (B), PstI; (C), BamHI; (D), HindIII; (E), EcoRI. The numbers at the left side indicate the size markers in kbp.

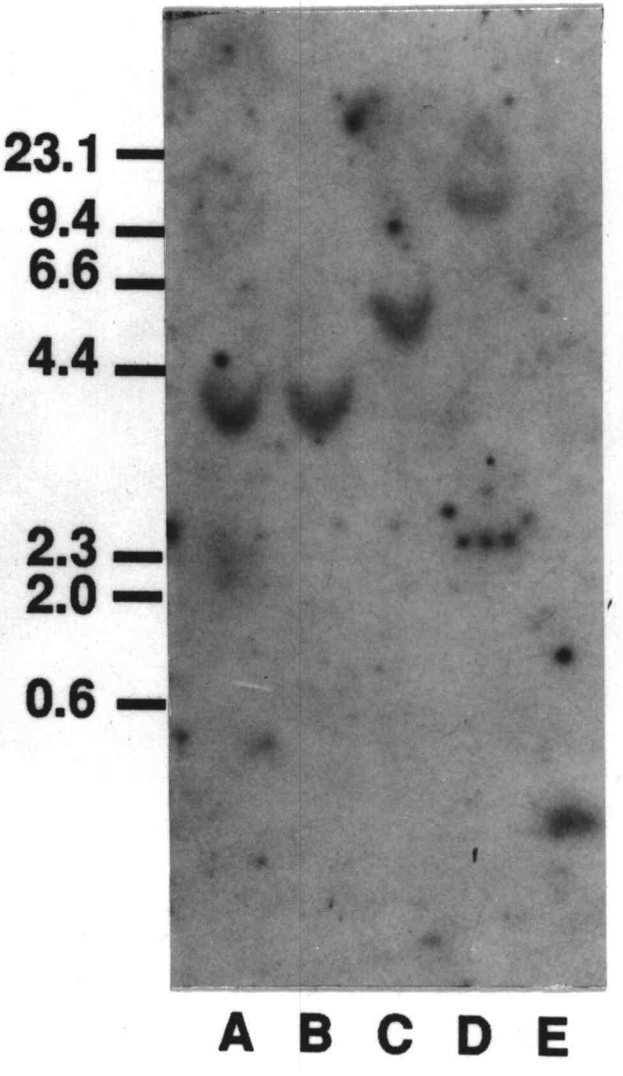

Figure 9

Figure 10: TGF-ß specificity of regulation of a brain RNA in SFME cells. SFME cells were grown in serum-free medium for 48 hours in the presence of (A) 10 ng/ml nerve growth factor (NGF), (B) 10 ng/ml platelet-derived growth factor (PDGF), (C) 10 ng/ml fibroblast growth factor (FGF), (D) 10 ng/ml TGF-ß or (E) without any additional peptide growth factor. Cytoplasmic RNA was extracted and Northern blot hybridization analysis was carried out using as probe a 3' HindIII fragment of a cDNA clone (B, see figure 17) identified as representing a TGF-ß regulated mRNA (20 μg RNA/lane).

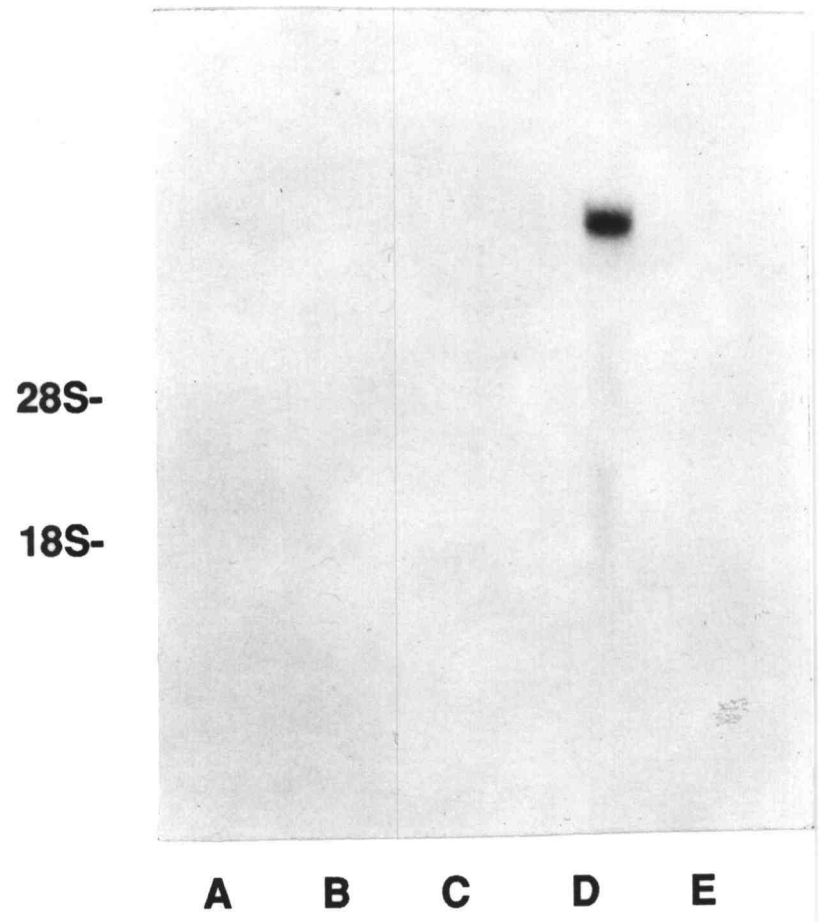

Figure 10

Figure 11: TGF-ß specificity of regulation of ß8. SFME cells were grown 48 hours in the presence of (A) 10 nM 3,5,3'-L-triiodothyronine, (B) 1ng/ml τ-interferon or (C) 10% CS. Cytoplasmic RNA was extracted and Northern blot hybridization analysis was carried out. Upper part: Probe was the 3' HindIII fragment of cDNA clone B (see figure 17), identified as representing a TGF-ß regulated RNA, lower part: same blot probed with ß-actin cDNA.

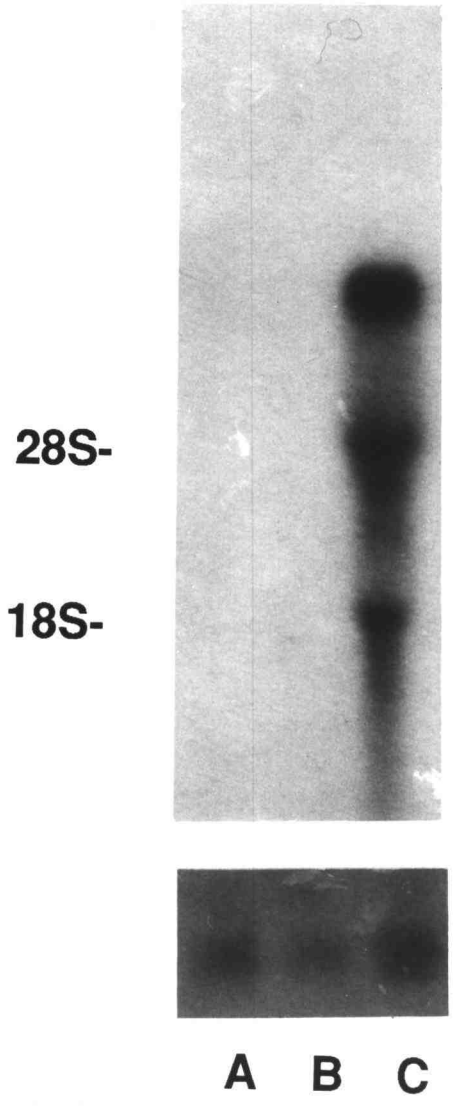

Figure 11

Figure 12: Time course of regulation of SFME cell ß8 RNA by TGF-ß. SFME cells were treated with TGF-ß (10ng/ml), cytoplasmic RNA was isolated and Northern hybridization blot analysis carried out using as probe the 3' HindIII fragment of cDNA clone B, identified as representing a TGF-ß regulated RNA. (A), no TGF-ß , 48 hour incubation; (B), TGF-ß 4 hours incubation; (C), TGF-ß 8 hours; (D), TGF-ß 24 hours; (E), TGF-ß 48 hours; (F), TGF-ß 24 hours, followed by 24 hour incubation without TGF-ß. Bottom: the same blot probed with radiolabeled ß-actin cDNA.

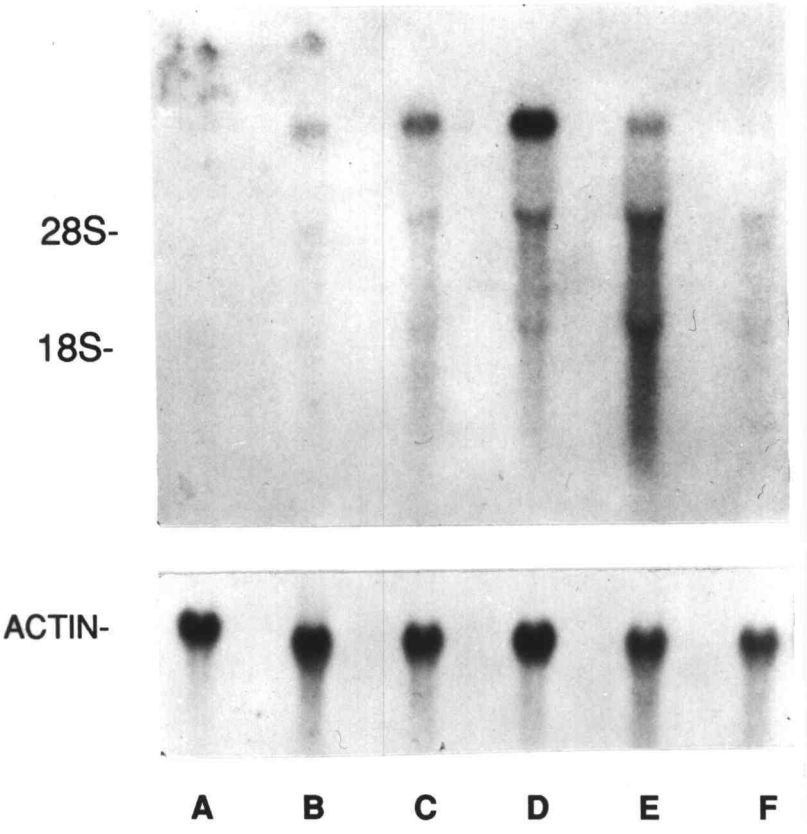

Figure 12

Figure 13: TGF-ß concentration dependence of regulation of ß8 RNA. SFME cells were treated with and without TGF-ß and cytoplasmic RNA was extracted after 24 hours, the Northern blot was probed with the 3' HindIII fragment of clone B (see figure 17), identified as representing a TGF-ß regulated RNA, and radiolabeled ß-actin cDNA. (A), 10 ng/ml TGF-ß; (B), 1 ng/ml TGF-ß; (C), 0.1 ng/ml TGF-ß; (D), 0.01 ng/ml TGF-ß; (E), no TGF-ß.

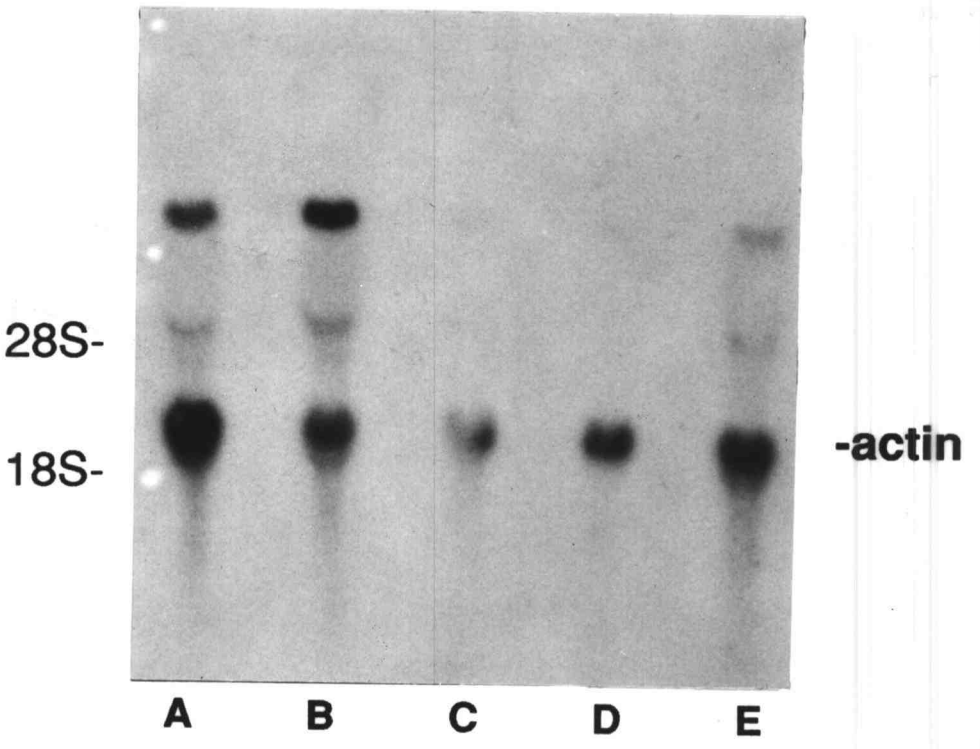

Figure 13

Figure 14: Stability of TGF-ß regulated RNAs in the presence of α-amanatin. Probe was the 3' HindIII fragment of clone B, identified as representing a TGF-ß regulated RNA in SFME cells. Top: (A), 0 hour incubation; (B) three hours incubation in the presence of TGF-ß and α-amanatin; (C), three hours α-amanatin incubation in the absence of TGF-ß; (D), six hours incubation in the presence of TGF-ß and α-amanatin; (E), six hours α-amanatin incubation in the absence of TGF-ß; (F), eight hours α-amanatin incubation in the presence of TGF-ß; (G), eight hours α-amanatin incubation in the absence of TGF-ß.

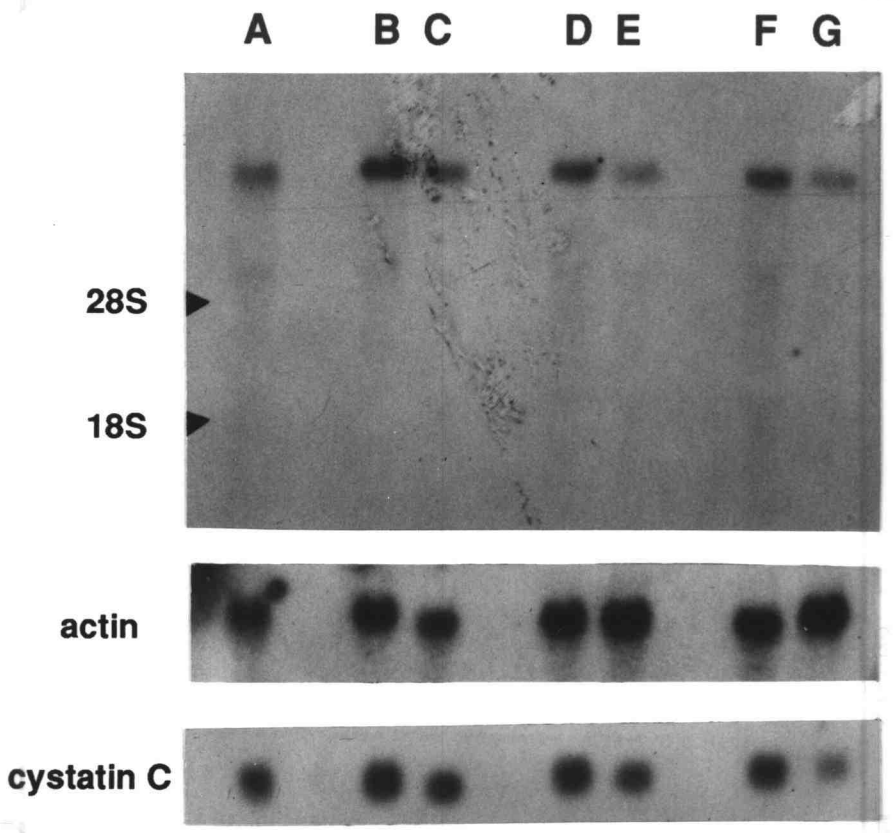

Figure 14

Figure 15: Cycloheximide effect on TGF-ß regulation of ß8 RNA. SFME cells were treated with TGF-ß (10 ng/ml) with or without cycloheximide (10 μg/ml), cytoplasmic RNA was analyzed in a Northern blot hybridization using as probe a cDNA piece of ß8 (3' HindIII fragment of clone B, see figure 17) and ß-actin. (A), cycloheximide and TGF-ß for 8 hours; (B), TGF-ß only for 8 hours; (C), cycloheximide only for 8 hours; (D), no TGF-ß or cycloheximide.

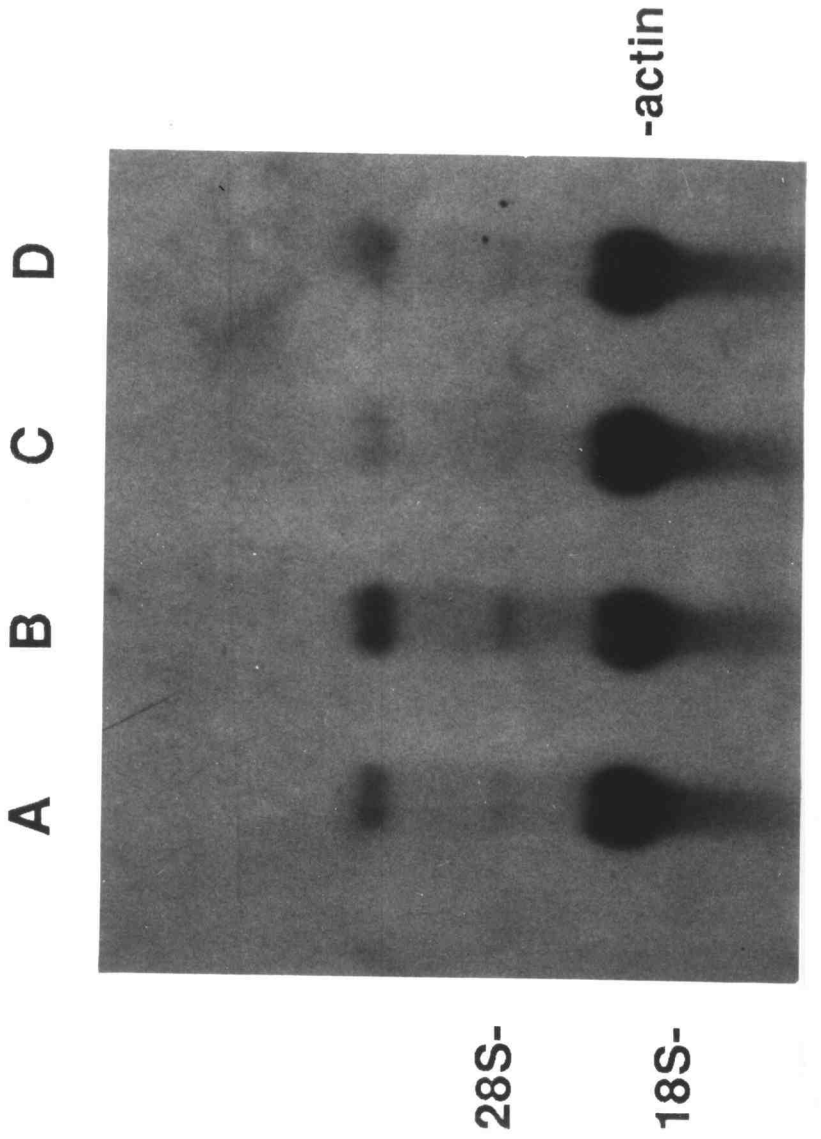

Figure 15

Figure 16: TGF-ß regulation of ß8 RNA in various transfected SFME cell lines. (A), SFME cells grown 24 h in the presence of 1 ng/ml TGF-ß; (B), neu-cl.2-1 SFME, no CS or TGF-ß; (C), neo-neu-cl.8, no CS or TGF-ß; (D), neo-SFME, no CS or TGF-ß; (E), Ha-ras-myc-cl.1 SFME, no CS or TGF-ß; (F),Ha-ras-cl.10 SFME, no CS or TGF-ß; (G), Ha-ras-cl.4 SFME, no CS or TGF-ß; (H), Ha-ras-cl.1 SFME, no CS or TGF-ß. Probe was the 3' HindIII fragment of clone B.

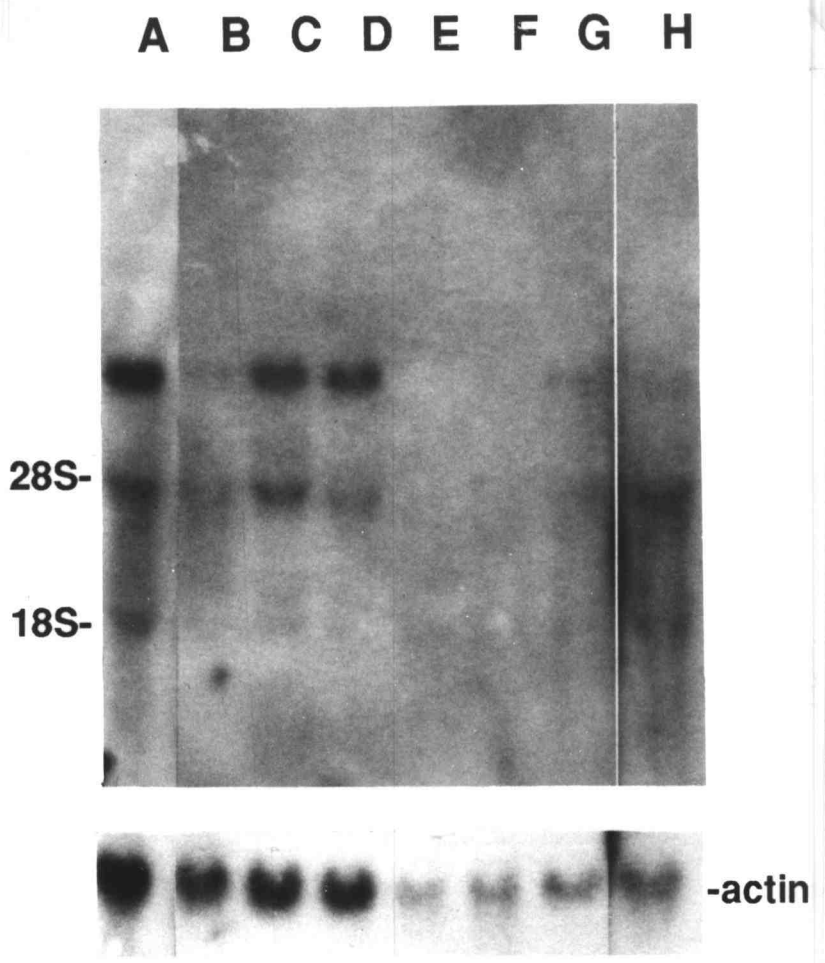

Figure 16

Figure 17: Map of cloned cDNA inserts representing ß8. 5' position is to the left, 3' to the right. Restriction sites, extend of overlaps and sequence features are indicated. Clones C and D are chimeras.

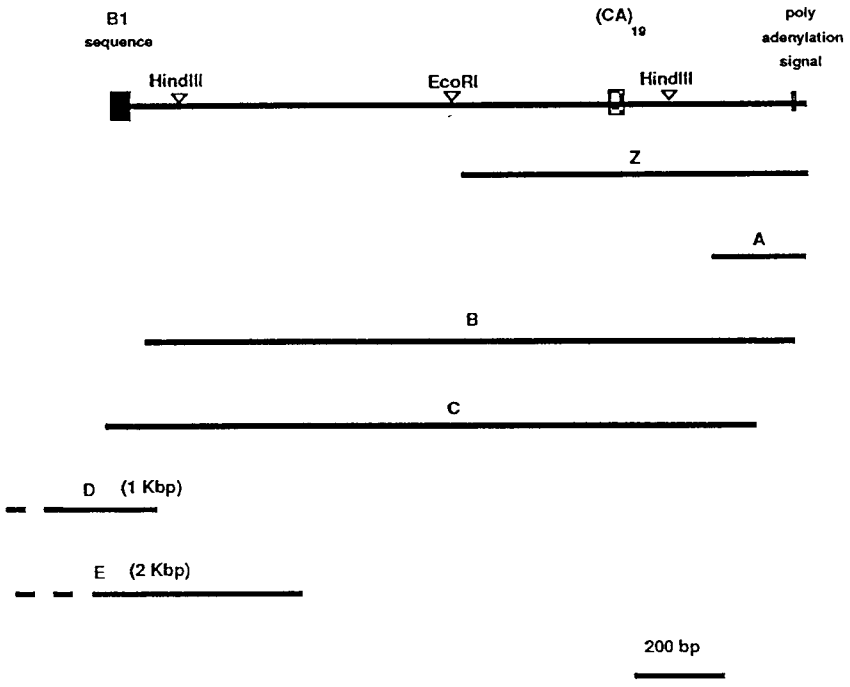
B1
sequence
(CA)19
poly
adenylation
signal
HindIII
EcoRI
HindIII
Z
A
B
C
D (1 Kbp)
E (2 Kbp)
200 bp

Figure 17

Figure 18: Partial cloned sequence of ß8. Part of the B1 sequence, the CA repeat, and the polyadenylation signal are underlined. Only sequence that was found in two independent phage clones is shown.

CTGGGATTAA AGGTGTGCGC CACCACGCCC GGCTCTACAT TTTTCAAAAT
GTGAAATAAT CCACTTTGGA TTCCCTCTTC CCATCTACAT CCCAGAAAAG
ACTTCCCAAG CCAGGGACAG GGTCCCACAG AGCCCATGCA GTGTGGGTAG
AATCTATGAA TTTGTGTTAA AGCCACAGTG GAGCCTAAGC TTAGCCCTGT
GGGTAAAGAT GCTGCATAAG CCTGAAGAAG TCCGTTTAAG CCCTGGGACC
CATGTTTTAA AAGTGAAAAG CCAGGCAGGG TGTGAACCCT GGTGCCAGCA
TCCCTGTGCT GAGACTATAG GAAGCTCATG AGCCAGCTAG CCTGGAGGAT
CACGGCAAAC CCTTAAAGGG TAGCATTTGA CCTCCACTGC ACACTACAGT
GTGGGTGATT CTCCAACACA CGCACAACCC TCTCCCCCCA CACACACTGC
ACAAGCACAA AATAAATAAG TAAAAATTTC AAAAACTGTA GGATTGGGTG
GGTCAGGGCA GGGATGGGTG CACAGGTTCT GGGGTCCATG CCCAGCTCTT
CCTAAGTGTT TACTGAGCTG CATTGCTCAC AGGAGGTAAT GAAAGCCTGG
GGCAGTTACT GAATAGAAGC CGTGTAAAGC CTTGTGAGAT GCCCTTGAAT
CCTCTCAGCA GCCCACGTGG TCCTTAGTCT CCCATTTCCA CTTGATTATA
AGAGAAAGAC AAAGTTACAG ACTACGATAG AGGAGCTAGA GCAAGAATTT
CCTTCTGTGC ATTATAATTA CTGTAAGTGT TGATTTTTTG GACAGGGCCT
CAGTCTGGCC TTGAATTCAC TCGGTAGCTG AGGATAACCT TAAAGTCTGA
TCCTTCTGCC TCAGTTTCCC AAGTACTAAA ACGGCAGGTA TGCACTATGA
CTGGTGTATA CAATGCTAGG GATGGAACCC AGAGATTTGT GCATACTAAG
TAAATGTTCT ACACCTGAGC TCCAAGTACT CTGTCAACTG AGCTCCAAGC
ACTCTGTCAA CTGAGCTACA AGTACTCTGT CAACTGAGCT CCAAGCACTC
TTATCTGTGC TTCGAACGCT ACCAACTTAG TTCGATTCGC AAACCCTGGT
CTTGATTGTG CTCCAAATTT TGCTTTATGT TTAAGGGAAG AGACCTAAAA
TCCTTACCTG CTGTCACACA CACACACACA CACACACACA CACACACACA
CACGGAGCAG ACTTGGGAAA TGGTTGAAAA AAGCAAACTG GACCCTGAAC
CGTAAAGGTC CCTGTGACTG GGGACAAAGC TTCTTGGTAA CATTTTCTAA
TTCAGAAGGA ACCACACCAA TCCCCTGCTG GGGATTGAGG GCGAGGCCTG
CTGGGGCTCA GAGGTTCTTC CTAGCCCCTG CACTGCCTGC TCCATGGAGG
GGCATGCTTT AGGCTTTTCT GGACTGGAGC CTCATCTCCA GCTTTCCCCC
TCCCTGGTGT GTCTGCTTGG GCCAGTATGC GGGAGGAGTG TGTATGTGCG
AGAATGGCTA CAGGTTCTGC TGGGTTTTGT TTTGTTTTTG TTTTTCTGGG
AGCTTTGATG TTCCGTGGAT CTGTGCTATC TGACACTGTG GATGTTAATG
GACTTATTGG ACATTTTAAT AAAAATTTTT TAACAGTTAA AAAAAAAAAA
AAAAAAAA

figure 18

Chapter IV

CONCLUSIONS AND PERSPECTIVES

SFME cells were derived in serum-free medium. The study of effects of serum on these cells lead to several discoveries: (i) The reversible growth inhibition of SFME cells by serum factors, (ii) the resistance of transformed SFME cells to this inhibition, (iii) the appearance of markers of differentiation in SFME cells when exposed to serum.

Plasma growth inhibitory activity. Growth inhibitory activity was also found in human plasma. The characterization and partial purification of the growth inhibitory activity of human plasma indicated that a macromolecular entity, probably a polypeptide, is a major contributor to the growth inhibitory activity. However, it seems that several components of the plasma interact to create the inhibitory effect of unfractionated plasma.

Most known growth inhibitory factors (e.g. TGF-ß) were not purified as growth inhibitors, but other activites associated with those factors were used to

identify and assay them, and their growth inhibitory activities were discovered later. The difficulties of interpreting growth inhibition assays and the possible artifactual effects make the direct purification of a growth inhibitor problematic, especially if one uses a complex mixture of substances such as plasma as starting material. It may, therefore, be useful to identify other activites that may be associated and copurify with the growth inhibitory activity. The striking morphological change of the SFME cells when exposed to the Sephadex G-50 purified preparation may lead to such a 'foothold'.

TGF-ß regulated brain RNA. Another effect of calf serum on SFME cells that was investigated in this thesis is the induction of a large, brain-specific message in SFME cells. This effect could be reproduced with TGF-ß, a serum component. This finding confirms the neuroectodermal origin of SFME cells and their in vitro differentiation under the influence of TGF-ß.

GFAP and cystatin C, a protease inhibitor, are other molecules that are regulated by TGF-ß in SFME cells. GFAP (Eng ,1988) and protease inhibitors (e.g. Wagner et al., 1989; Abraham et al., 1988) are associated with

pathological states of the brain. It is therefore not unreasonable to speculate that ß8 may be of clinical interest, too. It may therefore be important to characterize this message by cloning and sequencing more of the gene until a function can be attributed to it. The presence of repetitive elements in the gene may require the developement of special techniques to manage this task. Since repetitive elements are common in genes and mRNAs (maybe especially in the large mRNAs of the brain), such techniques could be of great general interest.

Serum and SFME. The multiple and complex effects serum has on SFME cells suggests physiological importance of serum or serum components in the regulation of the hypothetical *in vivo* counterparts of these cells. Defining the *in vivo* fates and properties of such cells may yield new and important insights.

REFERENCES

Abraham, C., Selkoe, D., Potter, H. (1988). Immunochemical Identification of the Serine Protease Inhibitor α_1-Antichymotrypsin in the Brain Amyloid Deposits of Alzheimer's Disease. Cell **52**, 487-501.

Ambesi-Impiombato, F. W., Parks, L. A. M., Coon, H. G.(1980). Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc. Natl. Acad. Sci. USA **77**, 3455-3459.

Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., Sporn, M. B. (1983). Transforming Growth Factor-ß in Human Platelets. J.B.C. **258**, No.11, 7155-7160.

Bains, W., Temple-Smith, K. (1989). Similarity and Divergence among Rodent Repetitive DNA Sequences. J. Mol. Evol. **28**, 191-199.

Bargmann, C. K., Hung, M. C., and Weinberg, R. A. (1986a). The neu oncogene encodes an epidermal growth factor-related protein. Nature, **319**, 226-230.

Bargmann, C. K., Hung, M. C., and Weinberg, R. A. (1986b). Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell, **45**, 649-657.

Barnes, D. W. (1987). Serum-Free Animal Cell Culture. BioTechniques Vol. **5**, No. 6, 534-542.

Barnes, D., Sato, G. H. (1980). Serum-free cell culture: A unifying approach. Cell **22**, 649-655.

Bjornson Childs, C., Proper, J. A., Tucker, R. F., Moses H. L. (1982). Serum contains a platelet-derived transforming growth factor. Proc. Natl. Acad. Sci. USA. **79**, 5312-5316.

Bressler, J. P.,and De Vellis, J. (1985). Neoplastic Transformation of Newborn Rat Astrocytes in Culture. Brain Research, **348**, 21-27.

Chang, Y.-J. (1988). Production of Peptide Growth Factors by Oncogene-Transformed Balb Serum-free Mouse Embryo Cells, Thesis.

Charrière-Bertrand, C., Garner, C., Tardy, M., Nunez, J. (1991). Expression of Various Microtubule-Associated Protein 2 Forms in the Developing Mouse Brain and in Cultured Neurons and Astrocytes. J. Neurochem. **56**, 385-391.

Cheifetz, S., Weatherbee, J. A., Tsang, M. L. S., Anderson, J. K., Mole, J. E., et al., (1987). The transforming growth factor-ß system, a complex pattern of cross-reactive ligands and receptors. Cell **48**, 409-415.

Chen, L., Mory, Y., Zilberstein, A., et al.,(1988). Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interferon-ß2. Proc. Natl. Acad. Sci. USA **85**, 8037-8041.

Couchie, D., Fages, C., Bridoux, A. M., Rolland, B., Tardy, M., Nunez, J. (1985). Microtubule-associated Proteins and In Vitro Astrocyte Differentiation. J. Cell Biol. **101**, 2095-2103.

David, I. B., Sargent, T. D., and Rosa, F.,(1990). The Role of Growth Factors in Embryonic Induction in Amphibians." Growth Factors and Development" (ed. Marit Nilsen-Hamilton) Current topics in Developmental Biology, Academic Press,Inc., Ca.

Davis, L. G., Dibner, M. D., Battey, J. F. (1986). Basic Methods in Molecular Biology, Elsevier, New York, Amsterdam, London.

De Vellis, J., Wu, D., & Kumar, S. (1986). ENZYME INDUCTION AND REGULATION OF PROTEIN SYNTHESIS. In "Biochemistry, Physiology, and Pharmacology of Astrocytes", eds. Federoff, S. & Vernadakis, A. (Academic, New York), Vol.**2**, 209-237.

Drew, P. D., and Ades I. Z. (1989). REGULATION OF THE STABILITY OF CHICKEN EMBRYO LIVER δ-AMINOLEVULINATE mRNA BY HEMIN. BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, **162**, 102-106.

Eng, L., Vanderhaegen, J., Bignami, A., Gerstl, B. (1971). An acidic protein isolated from fibrous astrocytes. Brain Res. **28**: 351-354.

Eng, L. F. (1988). Regulation of glial intermediate filaments in astrogliosis. in "The Biochemical Pathology of Astrocytes", editors Norenberg, M. D., Hertz, L., Schousboe, A.; Alan R. Liss, Inc., New York.

Frohman, M. A. (1990). RACE: RAPID AMPLIFICATION OF cDNA ENDS. in 'PCR Protocols', editors Innis, M. A., Gelfand, D. H., Sninsky J. J., White, T. J.; Academic Press, Inc., San Diego, New York, Boston, London, Sydney, Tokyo, Toronto.

Frohman, E. M., Van Den Noort, S., and Gupta, S. (1989). Astroctyes and Intracerebral Responses. J. Clinical Immunology, Vol.**9**, No.1, 1-9.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., Sporn, M. B. (1983). Purification and initial characterization of a type beta transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA **80**, 3676-3680.

Geisert, E. E., Johnson, H. G., Binder L. I. (1990). Expression of microtubule-associated protein 2 by reactive astrocytes. Proc. Natl. Acad. Sci. USA **87**, 3967-3971.

Graycar, J. L., Miller, D. A., Arrik, B. A., Lyons, R. M., Moses, H. L., Derynk, R. (1989). Human transforming growth factor-ß3: recombinant expression, purification and biological activities in comparison with transforming growth factors ß1 and ß2. Mol. Endocrinol. **3**, 1977-86.

Gubler, U. and Hoffman, B. J. (1983). Simple and very efficient method for generating cDNA libraries. Gene, **25**, 263-269.

Hamada, H., Petrino, M. G., and Kakunaga, T. (1982). A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proc. Natl. Acad. Sci. USA vol. **79**, 6465-6469

Heine, U. I., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Peter Lam, H.-Y., Thompson, N. L., Roberts, A. B., and Sporn, M. B. (1987). Role of Transforming Growth Factor-ß in the Development of the Mouse Embryo. The Journal of Cell Biology, Volume **105**, 2862-2875.

Hellman, L., Steen, M., Sundvall, M., and Pettersson, U. (1988). A rapidly evolvig region in the immunoglobulin heavy chain loci of rat and mouse: postulated role of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ sequences. Gene, **68**, 93-100.

Hertz, L., Juurlink, H. J., and Szuchet, S. (1982). Cell Cultures. 613-624; in "Handbook of Neurochemistry" vol. 8, ed. Lajitha, A., Plenum Press, New York and London.

Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H., and Sendtner, M. (1988). Cilliary neuronotrophic factor induces type-2 astrocyte differentiation in culture. Nature **335**, 70-73.

Kimelberg, H.,Norenberg, M. D.(1989). Astrocytes. Scientific American. April issue. 66-74.

Krayev, A. S., Kramerov, D. A., Skryabin, K. G>, Ryskov, A. P. Bayev, A. A. and Georgiev, G. P. (1980). The nucleotide sequence of the ubiquitous repetitive DNA sequence B1 complementary to the most abundant class of mouse fold-back RNA. Nucleic Acids Research, vol.**8**, no.6, 1201-1215.

Ito, F., Takii, Y., Suzuki, J., and Masamune, Y. (1982). Reversible inhibition by human serum lipoproteins of cell proliferation. J.Cell.Physiol., **113**, 1-13.

Jagus, R. (1987). Hybrid Selection of mRNA and Hybrid Arrest of Translation. in 'Methods in ENZYMOLOGY', vol.152, 567-572, editors Berger, S. L., Kimmel A. R., Academic Press, Inc., San Diego, New York, Berkely, Boston, London, Sydney, Tokyo, Toronto.

Jelinek, W. R. (1982). REPETITIVE SEQUENCES IN EUKARYOTIC DNA AND THEIR EXPRESSION. Ann. Rev. Biochem. 1982. **51**, 813-844.

Jennings, J. C., Mohan, S., Linkhart, T. A., Widstorm, R., Baylink, D. J. (1988). Comparison of the biological activities of TGF-ß1 and TGF-ß2: differential activity in endothelial cells. J. Cell Physiol. **137**, 167-172.

Juurlink, H. J., Hetz, L. (1985). Plasticity of Astrocytes in Primary Cultures: An Experimental Tool and a Reason for Methodological Caution. Devl.Neurosci. **7**: 263-277.

Lazarides, E. (1982). Intermediate Filaments. Annu. Rev. Biochem. **51**, 219-250.

Lewin, B. (1983). Genes II. John Wiley & Sons, N.Y., ppg.434.

Lillien, L. E., and Raff, M. C. (1990). Differentiation Signals in the CNS: Type-2 Astrocyte Development In Vitro as a Model System. Neuron, vol. **5**, 111-119.

Loo, D. T., Fuquay, J. I., Rawson, C. L., Barnes, D. W. (1987). Extended Culture of Mouse Embryo Cells without Senescence:Inhibition by Serum. Science **236**, 200-206.

Loo, D. T., Rawson, C. L., Ernst, T. Shirahata, S., Barnes, D. W. (1989a). Serum-free Primary Culture of Mouse Embryo Cells, in: Cell Growth and Cell Division: a Practical Approach, ed.R.Baserga, IRL press, pp.17-34.

Loo, D., Rawson, C., Helmrich, A., Barnes, D. (1989b). Serum-Free Mouse Embryo (SFME) Cells: Growth Responses in Vitro. J. Cell. Physiol. **139**, 484-491.

Loo, D., Rawson, C. Schmitt, M., Lindburg K. and Barnes, D. (1990). Glucocorticoid and thyroid hormones inhibit proliferation of serum-free mouse embryo (SFME) cells. J. Cell. Physiol. **142**, 210-217.

Maniatis, T., Fritsch, E. F., Sambrook, J. (1982). Molecular Cloning Manual. Cold Spring Harbor Press, New York.

Marx, J. (1991). How Embryos Tell Heads From Tails. Science **254**, 1586-1588.

Massagué, J. (1990). The Transforming Growth Factor-ß Family. Annu. Rev. Cell Biol. **6**, 597-641.

Masui, T., Wakefield L. M., Lechner J. F., LaVeck, M., Sporn, M. B. (1986). Type ß transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc. Natl. Acad. USA **83**, 2438-2442.

Mercola M.,Stiles, C. D. (1988). Growth factor superfamilies and mammalian embryogenesis Development **102**, 451-460.

Milner, R. J., Sutcliffe, J. G. (1983). Gene expression in rat brain. Nucleic Acids Research, vol. **11**, 16, 5497-5520.

Miyazaki K. and Horio T. (1989). Growth Inhibitors: Molecular Diversity and Roles in Cell Proliferation. In Vitro Cellular & Developmantal Biology vol.**25**, No.10 , 866-872.

Miyazono, K., Hellman, U., Wernstedt, C., Heldin, C.-H. (1988). Latent high molecular weight complex of transforming growth factor ß1. Purification from human platelets and structural characterization. J. Biol. Chem. **263**, 6407-6415.

Morrison, R. S., De Vellis, J., Lee, Y. L., Bradshaw, R. A., and Eng, L. F. (1985). Hormones and Growth Factors Induce the Synthesis of Glial Fibrillary Acidic Protein in Rat Brain Astrocytes. J. Neurosci. Res. **14**, 167-176.

Nieto-Sampedro M. (1988). Astrocyte Mitogen Inhibitor Related to Epidermal Growth Factor Receptor. Science, vol. **240**, 1784-1786.

Noble, M.m Murray, K., Stroobant, P., Waterfield, M. D.,and Riddle, P.(1988). Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature **333**, 560-562.

Ohta, M., Greenberger, J. S., Anklesaria, P., Bassols, A., Massagué, J. (1987). Two forms of transforming growth factor-ß distinguished by multipotential haematopoietic progenitor cells. Nature **329**, 539-41.

Ottmann, O. G., Pelus, L. M. (1988). Differential proliferative effects of transforming growth factor-ß on human hematopoietic progenitor cells. J. Immunol. **140**, 2661-65.

Pelton, R. W., Dickinson, M. E., Moses H. L., and Hogan B. L. M. (1990). *In Situ* hybridization analysis of TGFß3 RNA expression during mouse develpoment: comparative studies with TGFß1 and TGFß2. Develpoment **110**, 609-620.

Peristeris, P., Gaspar, A., Gros, P. (1988). Effects of serum amyloid A protein on lymphocytes, Hela, and MRC_5 cells in culture. Biochem. Cell. Biol. **67**, 365-370.

Prochiantz, A., Mallat, M. (1988). Astocyte Diversity. Ann. N. Y. Acad. Sci. **540,** 52-63.

Raff, M. C., Miller, R., Noble, M. (1983). A glial progenitor cell that developes *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. Nature vol. **303**, 390-396.

Rawson, C., Loo, D., Helmrich, A., Ernst, T.,Natsuno, T.,Merrill, G., Barnes, D. (1991). Serum Inhibition of Proliferation of Serum-Free Mouse Embryo Cells. Exp. Cell Res. **192**, 271-277

Roberts, A. B., Anzano, M. A., Lamb, L.C., Smith, J.M., and Sporn, M. B. (1981). New class of transforming growth factors potentiated by epidermal growth factor: Isolation from non-neoplasic tissues. Proc. Natl. Acad. Sci. USA **78**, 5339-5343.

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., et al., (1983). Purification and properties of a type beta transforming growth factor from bovine kidney. Biochemistry **22**, 5692-98.

Robertson, P. L., Markovac, J., Datta, S. C., Goldstein, G. W. (1988). Transforming growth factor beta stimulates phosphoinositol metabolism and translocation of protein kinase C in cultured astrocytes. Neuroscience Letters, **93**, 107-113.

Rothblat, G. H., Arbogast, L. Y., Ouèleette, L., Howard, B. V. (1976). Preparations of delipidized serum protein for use in cell culture systems. In Vitro **12**, 554-557.

Ruoslahti, E., Hayman, E., Peirschbacher, M., and Engvall, E. (1982). Fibronectin: purification, immunochemical properties and biological activities. Methods Enzymol., **82**, 803-830.

Saad, B., Constam, D. B., Ortmann, R., Moos, M., Fontana, A., Schachner, M. (1991). Astrocyte-derived TGF-ß2 and NGF Differentially Regulate Neural Recognition Molecule Expression by Cultured Astrocytes. J. Cell. Biol., **115**, 473-484.

Sakai Y.,Rawson C.,Lindburg K. and Barnes D. (1990). Serum and Transforming Growth Factor Beta Regulate Glial Fibrillary Acidic Protein in Serum-free Mouse Embryo Cells. Proc. Natl. Acad. Sci. USA **87**, 8378-8382.

Saneto, R. P., Altman, A., Knobler, R. L., Johnson, H. M., De Vellis, J. (1986). Interleukin 2 mediates the inhibition of oligodendrocyte progenitor cell proliferation <u>in vitro</u>. Proc. Natl. Acad. Sci. USA. **83**, 9221-9225.

Schluesener, H. J. (1990). Transforming growth factors type $beta_1$ and $beta_2$ suppress rat astrocyte autoantigen presentation and antagonize hyperinduction of class II major histocompatibility complex antigen expression by interferon- gamma and tumor necrosis factor-alpha . J. Neuroimmunol.; vol. **27**, no. 1, 41-47.

Shapiro, R. A., Farrell, L., Srinivasan, M. and Curthoys, N. P. (1991). Isolation, characterization, and in vitro expression of a cDNA that encodes the kidney isozyme of the mitochondrial glutaminase. J. Biol. Chem. **266**, 18792-18796.

Shirahata S.,Rawson C.,Loo D.,Chang Y., and Barnes D. (1990). Ras and Neu Oncogenes Reverse Serum Inhibition and Epidermal Growth Factor Dependence of Serum-free Mouse Embryo Cells. Cell Physiol. **144**, 69-76.

Slack, J. M.,(1990). Growth Factors as inducing agents in early Xenopus develpoment. J.Cell Sci. Suppl.B 119-130 (1990);

Solem, M., Rawson, C., Lindburg, K., and Barnes, D. (1990). TRANSFORMING GROWTH FACTOR BETA REGULATES CYSTATIN C IN SERUM-FREE MOUSE EMBRYO (SFME) CELLS. Biochem. Biophys. Res. Commun. **172**, no.2, 945-51.

Southern, P. J., and Berg, P. (1982). Transformation of Mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet., **1**, 327-341.

Sporn, M. B. and Roberts, A. B. (1988). Peptide growth factors are multifunctional. Nature **322**, 217-219.

Sugarman, B. J., Aggarwal, B. B., Hass, P. E., et al. (1985). Recombinant human tumor necrosis factor-α: effects on normal and transformed cells in vitro. Science **230**, 943-945.

Sutcliffe, J. G. (1988). mRNA IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM. Ann. Rev. Neurosci. 1988, **11**, 157-198.

Tsunawaki, S., Sporn, M. B., Ding, A., Nathan, C. (1988). Deactivation of macrophages by transforming growth factor-ß. Nature **334**, 260-362.

Ullu, E. and Tschudi, C. (1984). *Alu* sequences are processed 7SL RNA genes. Nature **312**, 171-172.

Wahl, S. M., Allen, J. B., McCartney-Francis, N., Morganti-Kossmann, M. C., Kossmann, T., Ellingsworth, L., Mai, U. E. H., Mergenhagen, S. E., Orenstein, J. M. (1991). Macrophage- and Astrocyte-derived Transforming Growth Factor ß as a Mediator of Central Nervous System Dysfunction in Acquired Immune Deficiency Syndrome. J. Exp. Med. **173**, 981-991.

Wagner, S. L., Geddes, J. W., Cotman, C. W., Lau, A. L., Gurwitz, D., Isackson, P. J., Cunningham D. D. (1989). Protease nexin-1, an antithrombin with neurite outgrowth activity, is reduced in Alzheimer disease. Proc. Natl. Acad. Sci. USA **86**, 8284-8288.

Wang, D., Lewis, S. A., Cowan, N. J. (1988). Complete sequence of a cDNA encoding mouse MAP2. Nucleic Acid Research **16**, no 23, 11369-11370.

Wilcox, J. N. and Derynk, R. (1988). Developmental expression of transforming growth factors alpha and beta in the mouse fetus. Molec. Cell. Biol. **8**, 3415-3422.