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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/00, 38/16, 38/18, 38/22, C07H 21/00, 21/04, C07K 14/00, 14/435, 14/475, 14/575, C12N 5/10, 5/16, 7/01, 15/00, 15/09, 15 /10, 15 /11, 15 /12, 15 /16, 15 /18, 15 /63, C12P 21 /00, 21 /02

A1

(11) International Publication Number: **WO 95/17203**

(43) International Publication Date: 29 June 1995 (29.06.95)

(21) International Application Number: PCT/US94/14771

(22) International Filing Date: 22 December 1994 (22.12.94)

(30) Priority Data:

08/172,327	22 December 1993 (22.12.93)	US
08/275,709	18 July 1994 (18.07.94)	US
08/359,480	20 December 1994 (20.12.94)	US

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.

(54) Title: NOVEL NUCLEIC ACID SEQUENCES ISOLATED FROM GLIAL CELLS

(57) Abstract

The present invention provides isolated astrocyte derived neurotrophic factor, cDNA encoding same, and uses thereof. The present invention further provides isolated nucleotide sequences which are preferentially expressed in T1 astrocytes, as well as nucleic acid sequences encoding conserved protein motifs and methods of using these sequences as markers of glial cell differentiation.

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Novel Nucleic Acid Sequences Isolated From Glial Cells

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention. NICHD HD 23315 and Javits award NINDS 10259.

Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Serial No. 08/275,709, filed July 18, 1994 which is a continuation-in-part of U.S. Serial No. 08/172,327, filed December 22, 1993, the content of both of which are hereby incorporated by reference.

Field of the Invention

The present invention relates to the field of neurobiology and neuropharmacology. The present invention discloses isolated neurotrophic agents, cDNA sequences encoding same, cDNA sequences for use as temporal regional and cell type markers, and uses of the novel neurotrophic agents of the present invention.

Related Art

Glial subtypes appear to play a critical role in brain function, elaborating growth and survival factors in a regionally-specific fashion (O'Malley, E.K. *et al., Exp. Neurol. 112*:40-48 (1991); O'Malley, E.K. *et al., Brain Res. 582*:65-70 (1992)). Moreover, emerging evidence suggests that local brain glia exert selective and specific effects on neuronal

architecture and function (Prochiantz, A. *et al.*, *Proc. Natl. Acad. Sci. USA* *76*:5387-5391 (1979)). However, while neuron typology is relatively well characterized, based on cytoarchitectonic, circuit and biochemical criteria, functional classification of regionally-specific glial subtypes is largely undefined. Nevertheless, development of a coherent glial biology requires specific and selective classification. An initial approach to define functional glial subpopulations by characterizing the spatiotemporal pattern of gene expression was developed.

While glia constitute approximately 90% of brain cellular elements, this widespread non-neuronal population had long been regarded as homogeneous. Classical histological studies had identified the astrocytes, oligodendrocytes and microglia, but further distinctions were unclear (Cajal, S.R., *Histologie du SystÅme Nerveux de l'Homme & des VertÄbrÄs*, Vol 1. L. Azoulay (trans) (1909). Madrid: Instituto Ramon y Cajal, 1952; Cajal, S.R., *Histologie du SystÅme Nerveux de l'Homme & des VertÄbrÄs*, Vol 2. L. Azoulay (trans) (1911). Madrid: Instituto Ramon y Cajal, 1955). More recently, glial subtypes were identified by detecting the selective expression of specific antigens. For example, GFAP (glial fibrillary acidic protein) was expressed by astrocytes, surface gangliosides were expressed by type 2 astrocytes and O-2A progenitors, and MBP (myelin basic protein) was expressed by oligodendrocytes (Raff, M.C., *Science 243*:1450-1455 (1989)).

Delineation of subtype functional specificity has paralleled immunologic classification. Oligodendrocytes have long been known to myelinate CNS axons, but the specific functions of type 1 and type 2 astrocytes have been more difficult to discern. Ultrastructural analysis of the optic nerve revealed that astrocytes exhibiting the type 2 phenotype send ramifying processes to the nodes of Ranvier (Fulton, B.P. *et al.*, *Ann. NY*

Acad. Sci. 633:27-34 (1991)). On the other hand, type 1 astrocytes appear to induce endothelial tight junctions, contributing to formation of the blood brain barrier (Janzer, R.C. *et al.*, *Nature 325*:253-257 (1987)). While these few examples indicate that classification has progressed in general, regional specificity, if existent, has yet to be extensively explored.

Recent evidence, however, does suggest that glial function differs on a regional as well as subtype-specific basis. In one example, mesencephalic type 1 astrocytes specifically and selectively elicit the survival of dopaminergic nigral neurons in cell culture (O'Malley, E.K. *et al.*, *Brain Res. 582*:65-70 (1992)). These and related observations have prompted the development of a new approach to glial classification, based on the spatiotemporal profile of the array of gene products expressed by any specific glial population.

The survival of ventral mesencephalic substantia nigra (SN) dopamine neurons, which degenerate in Parkinson's disease, is enhanced by glial cells in vitro. The recent isolation of glial cell line-derived neurotrophic factor (GDNF), a molecule with apparently selective effects on dopamine (DA) neurons in vitro, raises the question of whether this factor is found in normal brain cells. In this study, the polymerase chain reaction (PCR) was employed to determine the regional distribution and cellular localization of GDNF in the rat central nervous system.

It has recently been shown that specific local glial components elaborate factors that selectively increase the survival of ventral mesencephalic substantia nigra (SN) dopamine (DA) neurons in vitro (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)). Degeneration of these nigrostriatal DA neurons is the pathologic hallmark of Parkinson's disease (Hornykiewicz, O., *Pharmacol. Rev. 18*:925-964 (1966)). Although numerous hypotheses have

been proposed, the basis of this degeneration is unknown. The trophic effect of SN glia (O'Malley, E.K., *et al.*, *Exp. Neurol. 112*:40-48 (1991)), in particular Type 1 (T1) astrocytes (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)), on DA cells in vitro supports the hypothesis that degenerative disorders such as Parkinson's disease result from decreased availability, access, or responsiveness to specific survival factors (Appel, S.H., *Ann. Neurol. 10*:499-505 (1981); Carvey, P.M., *et al.*, *Exp. Neurol. 120*:149-152 (1993)). Identification of a glial-derived DA neuron survival factor may reveal potential pathogenetic mechanisms, and in turn, a more effective treatment.

Recently, a novel member of the transforming growth factor β (TGFβ) family, glial cell line-derived neurotrophic factor (GDNF), was purified from a rat cell line and the encoding gene was cloned from rat cDNA and human genomic DNA libraries (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)). Similar to the specific effect of SN T1 astrocytes (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)), GDNF appears to selectively enhance the survival of DA neurons in culture. However, purification from an immortalized cell line raises the question of whether GDNF is normally expressed in the central nervous system.

Summary of the Invention

In one aspect, the present invention is based on the identification, isolation, and characterization of a novel neurotrophic agent, a cDNA molecule encoding the novel neurotrophic agent, and methods of using the neurotrophic agent to stimulate neuronal cell survival and growth.

In detail, the present invention provides isolated astrocyte derived neurotrophic factor (hereinafter ATF). ATF comprises a family of proteins that includes ATF-1, ATF-2, and ATF-3. Preferably, ATF comprises the amino acid sequence depicted in SEQ ID NO:2 or SEQ ID NO:37 (rat ATF-1 and ATF-3) or in SEQ ID NO:31 or SEQ ID NO:33 (human ATFs, ATF-1 and ATF-2, respectively). One difference between the previously identified neurotrophic factor, GDNF (SEQ ID NO:35), and ATF is that certain centrally located amino acids of GDNF are deleted in ATF. The amino acid sequence of ATF-2 (SEQ ID NO:33) has a larger deletion than the amino acid sequence of ATF-1 (SEQ ID NO:31). The differences between GDNF and ATF generate a molecule (ATF) which differs in activity from GDNF in a cell type specific manner.

The present invention further provides isolated nucleic acid molecules which encode ATF. Using the universal codon table, a skilled artisan can readily determine all of the nucleic acid sequences which are capable of encoding ATF. The preferred nucleic acid sequence which encodes ATF are the naturally occurring human ATF encoding sequences, preferably ATF-1, ATF-2, and ATF-3 (for example, SEQ ID NO:30 and SEQ ID NO:32).

The nucleic acid sequence encoding ATF is expressed in a regional, developmental, and cell type specific fashion. Therefore, the present invention provides methods of assaying for the presence, in a sample, of a nucleic acid sequence encoding ATF. This method provides means for determining the cell type or developmental stage of a given cell.

Specifically, utilizing the nucleotide sequence of a ATF cDNA (for example, SEQ ID NO:30, SEQ ID NO:32, and SEQ ID NO:36), the present invention provides methods of identifying the presence of a nucleic acid

sequence in a sample which contains ATF encoding sequences comprising the steps of:

contacting a sample with two nucleic acid amplification primers, wherein said nucleic acid amplification primers are capable of hybridizing to the nucleic acid sequence encoding ATF, or a complementary sequence thereof;

amplifying the primed nucleic acid sequences in the sample; and

detecting the presence of amplified nucleic acid sequences in the sample which contains an ATF encoding nucleotide sequence.

In addition to methods which rely on the amplification of a target sequence, the present invention further provides methods for identifying nucleic acid sequences containing ATF encoding sequences which do not require sequence amplification. Specifically, the known methods of Southern and northern blot hybridization can be employed to determine if a sample contains ATF encoding nucleic acid sequences (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). In detail, ATF encoding nucleotide sequences can be detected by:

contacting a sample with a nucleic acid probe, wherein the probe consists essentially of the nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:30, SEQ ID NO:32 and SEQ ID NO:36, or a fragment thereof; and

detecting the presence of a nucleic acid sequence within the sample which hybridizes to said nucleic acid probe.

The materials used in the above assay methods are ideally suited for the preparation of a kit. For example, the invention provides a

compartmentalized kit to receive in close confinement, one or more containers which comprises:

(a) a first container comprising one or more of the amplification primers or probes of the present invention; and

(b) one or more other containers comprising one or more of the following: a sample reservoir, amplification reagents, wash reagents, and detection reagents.

The present invention further provides methods for using ATF to stimulate cell survival and growth. Specifically, ATF is effective in stimulating nerve cell survival and growth *in vitro* as well as *in vivo*. For *in vitro* use, ATF is useful in promoting survival and proliferation of cells in culture and for proliferating cells prior to transplantation or as sources for other novel neurotrophic agents. For *in vivo* use, ATF is useful in promoting survival and proliferation of cells and is useful in reducing the severity of pathological condition in which there is nerve degeneration and nerve cell death. Neuronal degeneration and death may be caused by physical damage to the brain, such as that caused by trauma, surgery, etc., or may also be caused by infection, nutritional deficiencies, and exposure to toxic agents. In addition, neuronal degeneration and death may be caused by neurodegenerative disorders such as stroke, Alzheimer's disease, Parkinson's disease, Huntington's Chorea, familial dysautonomia, Amyotrophic Lateral Sclerosis (ALS), olivopontocerebellar atrophy, spinal muscular atrophy, hereditary sensorimotor neuropathy and static encephalopathy (i.e. cerebral palsy). ATF can be used to reduce the severity of the symptoms of a wide range of those neuropathological states.

In a second aspect, the present invention provides the identification, isolation, and characterization of cDNA sequences which are complementary

to mRNA sequences which are expressed in a temporal, spatial, and cell specific fashion within the brain. These cDNAs are useful as markers of specific cell types and developmental stages of brain tissues.

Specifically, using a modified subtractive PCR procedure, several cDNAs have been isolated in which the mRNA corresponding to the cDNA is expressed at a higher level in T1 astrocytes when compared to the expression found in T2 astrocytes. These cDNAs, SEQ ID NO:3, 4, 28 and 29, have been characterized.

The present invention further provides cDNA sequences which encode important protein motifs which are associated with an important and conserved structural or biological activity. These cDNA sequences are useful in studying expression of these motifs, as sources of amplification primers and hybridization probes, and as sources, for epitopes useful in generating diagnostic and therapeutic antibodies.

Specifically, the present invention provides cDNA sequences, expressed in various brain tissues, which encode important structural or functional protein motifs which are present in transforming growth factor β1-3, G-TsF human glial blastoma-derived T-cell suppressor factor, and astrocyte derived neurotrophic factor. An example is provided in SEQ ID NO:5.

One nucleotide sequence of the present invention (SEQ ID NO:3) is derived from a rat source. However, due to the highly conserved nature of nucleotide sequences for brain specific transcripts and proteins with important biological function, the rat sequences can be used in human studies or can be used to isolate the human homologues of the sequences herein disclosed.

Brief Description of the Figures

Figure 1: PCR-based subtractive cloning methodology. Total RNA isolated from primary cultures enriched for type 1 or type 2 astrocytes was converted into double-stranded cDNA by established methods (Belyavsky, A., *Nuc. Acids Res. 17*:2919-2932 (1989); Brunet, J.F., *et al.*, *Science 252*:856-859 (1991)). The glial subtype-specific cDNA was utilized as template in asymmetric PCR's (McCabe, P.C., "Production of single-stranded DNA by asymmetric PCR," in *PCR Protocols: A Guide to Methods and Applications,* Innis *et al.*, (eds.), Academic Press, San Diego, CA, pp. 76-83 (1990)) to generate single-stranded cDNA for use as subtracting (type 2), or target cDNA populations (type 1). After stringent hybridization, single-stranded cDNA was collected by batch hydroxylapatite chromatography. Contaminating type 2 "U"DNA was removed by digestion of the eluted single-stranded fraction with uracil DNA glycosylase, followed by alkaline hydrolysis. The remaining type 1 cDNA typically represented 1-7% of the starting material determined by the amount of radioactivity remaining after the subtraction procedure. The final type 1-specific single-stranded cDNA was subjected to 15 cycles of PCR amplification to facilitate cloning into Not 1-digested pGEM5zf(+).

Figure 2. Comparison of rat GDNF and rat ATF cDNA sequences (SEQ ID NO:26, lower sequence and SEQ ID NO:1, upper sequence, respectively).

Figure 3. Comparison of rat GDNF and rat ATF peptide sequence (SEQ ID NO:27, lower sequence and SEQ ID NO:2, upper sequence, respectively).

Figure 4. Schematic comparison of human GDNF, ATF-1 and ATF-2.

Figure 5. Ethidium bromide stained agarose gel depicting PCR analysis of GDNF/ATF RNA. Lane 1: Lambda BstE II molecular weight markers. Lane 2: Mock PCR reaction with reverse transcriptase but without RNA template. Lane 3: PCR reaction with 50 ng rat brain genomic DNA. Lane 4: PCR reaction using RNA isolated from Rat cultured type 1 astrocytes (GDNF amplification was greater than ATF amplification). Lane 5: PCR reaction using isolated human SN RNA. Lane 6: PCR reaction using human caudate RNA (ATF amplification was greater than GDNF amplification). Lane 7: PCR reaction using human putamen RNA (low levels of GDNF and ATF amplified).

Detailed Description of the Preferred Embodiments

In one aspect, the present invention is based on the identification, isolation, and characterization of a novel neurotrophic agent, cDNA molecule encoding a novel neurotrophic agent, and methods of using the neurotrophic agent to stimulate neuronal cell survival and growth.

In detail, the present invention provides isolated astrocyte derived neurotrophic factor (hereinafter ATF). ATF comprises a family of proteins that includes ATF-1, ATF-2, and ATF-3. Preferably, ATF comprises the amino acid sequence depicted in SEQ ID NO:2 and SEQ ID NO:37 (rat ATF-1 and ATF-3) and in SEQ ID NO:31 and SEQ ID NO:33 (human ATFs, ATF-1 and ATF-2). One difference between the previously identified neurotrophic factor, GDNF (SEQ ID NO:35), and ATF is that certain centrally located amino acids of GDNF are not present in ATF. The amino

acid sequence of ATF-2 (SEQ ID NO:33) has a larger deletion than the amino acid sequence of ATF-1 (SEQ ID NO:31) and the first 18 amino-acids differ. The differences between GDNF and ATF generate a molecule (ATF) which differs in activity from GDNF in a cell type specific manner. The present invention is intended to encompass all ATF proteins with the characteristics of ATF which differ from GDNF. A comparison of the nucleic acid and amino acid sequences of rat ATF and rat GDNF is provided in Figures 2 and 3, respectively. The differences in amino acid sequence generate a molecule which differs in activity from GDNF in a cell type specific manner. Specifically, experiments determined that both GDNF and ATF were active molecules. In the basal forebrain both GDNF and ATF application resulted in a similar significant decrease in activity of the acetylcholine synthesizing enzyme, choline acetyltransferase. In addition, in the substantia nigra, both factors elicited significant increases in the activity of the catecholamine enzyme tyrosine hydroxylase (TH).

As used herein, ATF is said to be isolated if a physical or chemical separation technique is employed to purify ATF above the level of purity in which ATF is found to exist in nature. Methods for isolating a protein with a known amino acid sequence are well known in the art. These include, but are not limited to, chromatographic separation based on charge, size, hydrophobicity, or immunological reactivity, and physical separation based on sedimentation coefficient and size. A skilled artisan can readily adapt known procedures for isolating a protein in order to obtain the isolated ATF of the present invention.

The ATF of the present invention can be isolated from either cells which naturally express ATF, from cells which have been altered so as to express ATF or can be chemically synthesized using known methods. A

skilled artisan can use any of a variety of these techniques so long as the isolated ATF consists essentially of the amino acid sequence depicted in SEQ ID NO:2 or SEQ ID NO:37 (rat ATF-1 and ATF-3) or in SEQ ID NO:31 or SEQ ID NO:33 (human ATFs, ATF-1 and ATF-2).

The present invention further provides isolated nucleic acid molecules which encodes ATF, preferably, ATF-1, ATF-2, and ATF-3. Using the universal codon table, a skilled artisan can readily determine all of the nucleic acid sequences which encode a protein with the amino acid sequence of ATF. The preferred nucleic acid sequence is the naturally occurring ATF encoding sequence. The rat ATF-1 and ATF-3 sequences are depicted in SEQ ID No. 1 and SEQ ID NO:36. Preferred nucleic acid sequences which encode ATF are the naturally occurring human ATF encoding sequences, ATF-1, ATF-2, and ATF-3 (preferably, SEQ ID NO:30 and SEQ ID NO:32).

By inserting a nucleic acid sequence which encodes ATF into an appropriate vector, one skilled in the art can readily produce large quantities of the ATF nucleotide sequence. These sequences can then be used to generate detection probes or amplification primers. Alternatively, an isolated nucleic acid sequence encoding ATF can be inserted into an expression vector. By introducing an expression vector containing ATF sequences into an appropriate host, ATF can be produced in large amounts.

There are numerous host/vectors systems available for the propagation of nucleic acid sequences and/or the production of recombinantly expressed protein. These include, but are not limited to, plasmid, viral and insertional vectors, and prokaryotic and eukaryotic host. One skilled in the art can readily adapt any host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

The present invention further provides evidence that the nucleic acid sequence encoding ATF is expressed in a regional, developmental, and cell type specific fashion. Based on this observation, the present invention provides methods of assaying for the presence, in a sample, of a nucleic acid sequence encoding ATF. This method provides a means for determining the cell type or developmental stage of a given cell.

One example of an assay method of the present invention which is used to detect ATF encoding sequences is based on the preferential amplification of nucleic acid sequences within a sample which encode ATF.

In general, an amplification reaction, such as the polymerase chain reaction (PCR), is used to amplify either the mRNA encoding ATF, or the genomic ATF gene. Specifically, utilizing the nucleotide sequences of the ATF cDNA (SEQ ID NO:1, 30, 32 or 36), the present invention provides methods of identifying the presence of a nucleic acid sequence in a sample which contains ATF encoding sequences comprising the steps of:

contacting a sample with two nucleic acid amplification primers, wherein said nucleic acid amplification primers are capable of hybridizing to the nucleic acid sequence encoding ATF, or a complementary sequence thereof;

amplifying the primed nucleic acid sequences in the sample; and

detecting the presence of amplified nucleic acid sequence in the sample which contains an ATF encoding nucleotide sequence.

As used herein, an amplification primer is any short DNA sequence which can hybridize to a target nucleic acid sequence and allow the target sequence to be amplified when incubated with the appropriate reagents under the appropriate conditions, for example, see Ausubel *et al.*, *Current Protocols*

in Molecular Biology, Wiley Press (1993). Amplification requires the use of two primers which flank the region which is to be amplified. One primer hybridizes to the target sequence while the other primer hybridizes to a sequence complementary to the target sequence. In the present invention, the amplification primers are derived from the nucleotide sequence depicted in SEQ ID NO:1, 30, 32, or 36. Any fragment of SEQ ID NO:1, 30, 32, or 36 can be used to generate appropriate amplification primers so long as the fragment which is chosen yields a suitable primer. Preferred amplification primers are designed so a skilled artisan can detect differences amongst sequences which encode ATF from sequences which encode similar but distinguishable proteins such as GDNF.

The target nucleic acid sequence which is to be amplified can either be the mRNA which encodes ATF or can be genomic DNA which contains the ATF gene. A skilled artisan can readily employ techniques known in the art to prepare a sample containing the appropriate target molecule. (*PCR Protocols*, Cold Spring Harbor Press (1991)).

As used herein, an amplification primer is said to be capable of hybridizing to a nucleic acid sequence if the primer is capable of forming hydrogen bonds with the target sequence under appropriate condition. In general the preferred condition are characterized as being high stringency condition. A skilled artisan can readily determine the appropriate conditions following methods described elsewhere (*PCR Protocols*, Cold Spring Harbor Press (1991), Privitera *et al., Blood 79*:1781 (1992)).

As used herein, amplification refers to the process of generating multiple copies of a target sequence. Various methods and enzymes are available to accomplish this goal. In the preferred embodiment, *Taq* DNA polymerase is used in the method known as PCR to amplify the target

sequence (see Example 1). However, a skilled artisan can substitute other enzymes for the *Taq* polymerase so long as the amplification goal is achieved.

As used herein, detecting the amplified target sequence refers to any method which can be employed to determine the presence or absence of an amplified nucleic acid sequence of a given size or of a particular nucleotide sequence. In one application, the amplification product is subjected to agarose or acrylamide gel electrophoresis to resolve the various sizes of nucleic acids which are present in the amplified sample. The resulting gel is then analyzed visually using a nucleic acid stain, for example ethidium bromide, to determine if an appropriately sized nucleic acid molecule is present in the amplified sample.

Alternatively, a detectably labeled probe can be employed to determine if the sample contains the amplified sequence. Such a probe can be used following the above described electrophoresis, or can be used in a dot blot or in situ assay method. The preferred amplification detection methods allows a skilled artisan to differentiate sequences which encode ATF from sequences which encode closely related proteins such as GDNF.

In addition to methods which rely on the amplification of a target sequence, the present invention further provides methods for identifying nucleic acids containing ATF encoding sequences which do not require sequence amplification. Specifically, the known methods of Southern and northern blot hybridization can be employed to determine if a sample contains ATF encoding nucleic acid sequences (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). In detail, ATF encoding nucleotide sequences can be detected by:

contacting a sample with a nucleic acid probe, wherein the probe consists essentially of the nucleotide sequence depicted in SEQ ID NO:1, 30, 32 or 36, or a fragment thereof; and

detecting the presence of a nucleic acid sequence within the sample which hybridizes to said nucleic acid probe.

The nucleic acid probes of the present invention include DNA as well as RNA probes. Such probes can be generated using techniques known in the art (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). A skilled artisan can employ such known techniques using the ATF cDNA sequences herein described, or fragments thereof, to generate suitable hybridization probes. The preferred hybridization detection methods of the present invention allows a skilled artisan to differentiate sequences which encode ATF from sequences which encode closely related proteins such as GDNF.

Any method known in the art can be utilized to label the probes used in the above assay methods. These include, but are not limited to, radioisotopes, enzymes or chromophores.

The samples used in the detection methods of the present invention include, but are not limited to, cells or tissues, protein, membrane, or nucleic acid extracts of the cells or tissues, and biological fluids such as cerebral fluid, blood, serum, and plasma. The sample used in the above-described method will vary based on the assay format, nature of the detection method, and the tissues, cells or extracts which are used as the sample. Methods for preparing protein extracts, membrane extracts or nucleic acid extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the method utilized. One preferred type of

sample which can be utilized in the present invention is derived from isolated neuronal tissues and cells. Such cells can be used to prepare a suitable extract or can be used in procedures based on *in situ* analysis.

The materials used in the above assay methods are ideally suited for the preparation of a kit. For example, the invention provides a compartmentalized kit to receive in close confinement, one or more containers which comprises:

(a) a first container comprising one or more of the amplification primers or probes of the present invention; and

(b) one or more other containers comprising one or more of the following: a sample reservoir, amplification reagents, wash reagents, and detection reagents.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the antibodies or probes used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or the hybridized probe.

For nucleic acid probes, examples of detection reagents include, but are not limited to radiolabeled probes, enzymatic labeled probes (horse radish peroxidase, alkaline phosphatase), and affinity labeled probes (biotin, avidin,

or steptavidin). For antibodies, examples of detection reagents include, but are not limited to, labelled secondary antibodies, or in the alternative, if the primary antibody is labelled, the chromophoric, enzymatic, or antibody binding reagents which are capable of reacting with the labelled antibody. One skilled in the art will readily recognize that the antibodies and nucleic acid probes described in the present invention can readily be incorporated into one of the established kit formats which are well known in the art.

The present invention further provides methods for using ATF to stimulate cell survival and growth. Specifically, ATF is effective in stimulated nerve cell survival and growth *in vitro* as well as *in vivo*. For *in vitro* use, ATF is useful in promoting survival and proliferation of cells in culture and for proliferating cells prior to transplantation. For *in vivo* use, ATF is useful in promoting survival and proliferation of cells and is useful in reducing the severity of pathological condition in which there is nerve degeneration and nerve cell death. Neuronal degeneration and death may be caused by physical damage to the brain, such as that caused by trauma, surgery, etc., or may also be caused by infection, nutritional deficiencies, and exposure to toxic agents. In addition, neuronal degeneration and death may be caused by neurodegenerative disorders such as stroke, Alzheimer's disease, Parkinson's disease, Huntington's Chorea, familial dysautonomia, Amyotrophic Lateral Sclerosis (ALS), olivopontocerebellar atrophy, spinal muscular atrophy, hereditary sensorimotor neuropathy and static encephalopathy (i.e. cerebral palsy).

The ATF of the present invention is preferably administered to a mammal intracerebrally via intercisternal or transcranial injection. When administering ATF by injection, the administration may be by continuous injections, or by single or multiple injections.

The ATF of the present invention is intended to be provided to recipient mammal in a "pharmaceutically acceptable form" in an amount sufficient to "therapeutically effective." An amount is said to be therapeutically effective if the dosage, route of administration, etc. of the agent are sufficient to increase the rate of survival of nerve cells or to induce a physiological response which is mediated by ATF. ATF is said to be "pharmacologically acceptable form" if its administration can be tolerated by a recipient patient.

The ATF of the present invention can be formulated according to known methods of preparing pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A., Ed., Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition which is suitable for effective administration, such compositions will contain an effective amount of an antibody of the present invention together with a suitable amount of carrier.

In providing a patient with ATF, the dosage of administered ATF will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of ATF which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

The ATF of the present invention may be administered alone, or in combination with another agent which has mitogenic or neurotrophic activity. Factors which are useful in such a combination include those which act

generally as mitogens and/or trophic factors for a variety of neuronal cell types. Factors which act as mitogens include, but are not limited to, insulin, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), epidermal growth factor (EGF), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), and interferon, VIP, bFGF, insulin, IGF-I, IGF-II, EGF, PACAP, brain derived neurotrophic factor (BDNF), and somatostatin. Trophic factors which are useful in the above described co-administration, include, but are not limited to, neurotrophin-3 (NT3), nerve growth factor (NGF), VIP, PACAP, basic fibroblast growth factor (bFGF), insulin, IGF-I, IGF-II, interferon, ciliary neurotrophic factor (CNTF). Growth factors include, but are not limited to, nerve growth factor and related neurotrophins (such as brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and neurotrophin-5 (NT5)), basic and acidic fibroblast growth factor (aFGF and bFGF), and transforming growth factors(such as TGF-βs), glial derived neurotrophic factor (GDNF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, insulin-like growth factors, transforming growth factor α (TGF-α), leukemia inhibitory factor (LIF), interleukins, interferons, and colony stimulating factors (CSFs)).

The methods of the invention may also be applied *in vivo* to treat neuronal degeneration and death by inducing ATF expression in the brain or by introducing cells expressing ATF into the brain which can then supply ATF to surrounding neurons.

The present invention provides the identification, isolation, and characterization of cDNA sequences which are complementary to mRNA sequences encoding important or evolutionary important protein motifs which are expressed in a temporal, spatial, and cell specific fashion within the brain.

These cDNAs are useful as markers of specific cell types and developmental stages of brain tissues and in the production of diagnostic and therapeutic antibodies.

Specifically, using a modified subtractive PCR procedure, several cDNAs have been isolated in which the mRNA corresponding to the cDNA is expressed at a higher level in T1 astrocytes when compared to the expression found in T2 astrocytes. These cDNAs, SEQ ID NO:3, 4, 28 and 29, have been characterized. Based on these observation, the present invention provides isolated nucleic acid molecules which consist essentially of a nucleotide sequence selected from the group consisting of SEQ ID NO:3, 4, 28 and 29.

In a third aspect of the present invention, a novel approach was employed to isolated cDNA sequences which encode proteins which have important biological activity within the brain. In detail, amplification primers were selected which corresponded to common elements found in the transforming growth factor beta family of proteins, (TGF-beta 1-3), and ATF and GDNF (SEQ ID NO:16 - 19). The TGF-beta family is comprised of a number of proteins that have mitogenic, inhibitor and/or transforming activity. These proteins have not been shown to be expressed in the brain. However, nucleic acid and/or protein sequence motifs which are evolutionarily conserved have been shown to correlate with structural or functional important activities. Sequences conserved amongst proteins with different regions of expression can further correspond to important functional and structural features of the protein.

Using sequence homologies found between TGF-beta family members, ATF and GDNF, nucleic acid sequences encoding important glial proteins have been identified, isolated and characterized (Seq. ID. No 5-11).

As used herein, a nucleic acid molecule is said to be isolated if the molecule has been treated such that it is substantially free from the nucleic acid sequences it is associated with in nature. The level of purity will vary depending on the intended use. For use as a hybridization probe or amplification primer, the isolated nucleic acid molecule will be substantially free from all other nucleic acid sequences. For other intended uses, a lower level of purity may be sufficient.

By inserting any of the nucleic acid sequences of the present invention into an appropriate vector, one skilled in the art can readily produce large quantities of the specific sequence. Alternatively, the nucleic acid sequences of the present invention can be inserted into an expression vector in order to produce the amino acid sequences of the present invention.

There are numerous host/vectors systems available for the propagation of nucleic acid sequences and/or the production of expressed proteins. These include, but are not limited to, plasmid and viral vectors, and prokaryotic and eukaryotic host. One skilled in the art can readily adapt any host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

The nucleic acid sequences of the present invention encode proteins which have important biological activities to which a significant amount of pharmaceutical development can be addressed. To facilitate this development, and as means of further characterizing the proteins encoded by the nucleic acid sequences of the present invention, the present invention provides antibodies which bind to important epitopes. Specifically, a skilled artisan can use known procedures to obtain antibodies which bind to the various proteins encoded by the sequences of the present invention (SEQ ID NO:3-11, 28 and 29) (for example see Harlow, *Antibodies* Cold Spring Harbor Press

(1989)). A skilled artisan can use these antibodies to purify and characterize the proteins encoded by SEQ ID NO:3-11, 28 and 29, or as therapeutical inhibitors (binding agent) of the protein which they are directed to.

In Example 2, the present invention provides evidence that the nucleic acid sequences of the present invention (SEQ ID NO:3, 4, 28 and 29) are expressed in a regional, cell type specific, and developmental specific fashion. In example 3, the present invention provides evidence that the nucleic acid sequences of the present invention (SEQ ID NO:5-11) encode proteins with important peptide sequence motifs. Some of these sequences are expressed in a regional, cell type specific, and developmental specific fashion. Based on these observation, the present invention provides methods of assaying for the presence, in a sample, of a nucleic acid sequence which is complementary to one of the cDNA sequences herein described. This method therefore provides a means for determining the cell type or development stage of a given cell, or whether a given sequence is expressed in a particular cell.

One example of the assay methods of the present invention which are used to detect sequences complementary to the sequences of the present invention are based on the preferential amplification of sequences within a sample which contains one of the nucleic acid sequences of the present invention.

In general, an amplification reaction such as the polymerase chain reaction (PCR) is used to amplify either mRNA complementary to the cDNA sequences of the present invention, or the genomic DNA which contains the genomic equivalents of the sequences of the present invention. Specifically, utilizing the nucleic acid sequences herein described, the present invention provides methods of identifying the presence of a nucleic acid sequence in a sample which contains sequences complementary to a sequence selected from

the group consisting of SEQ ID NO:3 - 11, 28 and 29, comprising the steps of:

contacting a sample with two nucleic acid amplification primers, wherein said nucleic acid amplification primers are capable of hybridizing to the nucleic acid sequence selected from the group consisting SEQ ID NO:3-11, 28 and 29;

amplifying the primed nucleic acid sequences in the sample; and

detecting the presence of an amplified nucleic acid sequence in said sample which contains sequences complementary to a nucleotide sequence selected from the group consisting of SEQ ID NO:3-11, 28 and 29.

As used herein, an amplification primer is any short DNA sequence which can hybridize to a target nucleic acid sequence and allow the target sequence to be amplified when incubated with the appropriate reagents under the appropriate condition. For example see Ausubel *et al.*, *Current Protocols in Molecular Biology*, Wiley Press (1993). Amplification requires the use of two primers which flank the region which is to be amplified. One primer hybridizes to the target sequence while the other primer hybridizes to a sequence complementary to the target sequence. In the present invention, the amplification primers are derived from the nucleotide sequence depicted in SEQ ID NO:3-11, 28 and 29. Any fragment of SEQ ID NO:3-11, 28 and 29, can be used to generate appropriate amplification primers so long as the fragment which is chosen yields a suitable primer.

The target nucleic sequence which is to be amplified can either be the mRNA which is complementary to a sequence selected from SEQ ID NO:3-11, 28 and 29 or can be genomic DNA which contains the sequence. A skilled artisan can readily employ techniques known in the art to prepare a sample containing the appropriate target molecule.

As used herein, an amplification primer is said to be capable of hybridizing to a nucleic acid sequence if the primer is capable of forming hydrogen bonds with the target sequence under appropriate condition. In general the preferred condition are characterized as being high stringency condition. A skilled artisan can readily determine the appropriate conditions following methods described elsewhere (*PCR Protocols*, Cold Spring Harbor Press (1991), Privitera *et al., Blood 79*:1781 (1992)).

As used herein, amplification refers to the process of generating multiple copies of a target sequence. Various methods and enzymes are available to accomplish this goal. In the preferred embodiment, *Taq* DNA polymerase is used in the method known as PCR to amplify the target sequence. However, a skilled artisan can substitute other enzymes for the *Taq* polymerase so long as the amplification goal is achieved.

As used herein, detecting the amplified target sequence refers to any method which can be employed to determine the presence or absence of an amplified nucleic acid sequence of a given size or a particular sequence. In one application, the amplification product is subjected to agarose or acrylamide gel electrophoresis to resolve the various sizes of nucleic acids which are present in the amplified sample. The resulting gel can then be analyzed visually using a nucleic acid stain, for example ethidium bromide, to determine if an appropriately sized nucleic acid molecule is present in the amplified sample.

Alternatively, a detectably labeled probe can be employed to determine if the sample contains the amplified sequence. Such a probe can be used following the above described electrophoresis, or can be used in a dot blot or in situ assay method.

In addition to methods which rely on the amplification of a target sequence, the present invention further provides methods for identifying nucleic acids containing a sequence selected from SEQ ID NO:3-11, 28 and 29, which do not require sequence amplification. Specifically, the known methods of Southern and Northern blot hybridization can be employed to determine if a sample contains the desired nucleic acid sequence (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). In detail, SEQ ID NO:3-11, 28 and 29, can be detected by:

contacting a sample with a nucleic acid probe, wherein the probe is selected from the group consisting of SEQ ID NO:3-11, 28 and 29, or a fragment thereof,; and

detecting the presence of a nucleic acid sequence within the sample which hybridizes to said nucleic acid probe.

The nucleic acid probes of the present invention include DNA as well as RNA probes. Such probes can be generated using techniques known in the art (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). A skilled artisan can employ such known techniques using the nucleotide sequences herein described, or fragments thereof, to generate suitable hybridization probes.

Any method known in the art can be utilized to label the probes used in the above assay methods. These include, but are not limited to, radioisotopes, enzymes or chromophores.

The samples used in the detection methods of the present invention include, but are not limited to, cells or tissues, protein, membrane, or nucleic acid extracts of the cells or tissues, and biological fluids such as blood, serum,

and plasma. The sample used in the above-described method will vary based on the assay format, nature of the detection method, and the tissues, cells or extracts which are used as the sample. Methods for preparing protein extracts, membrane extracts or nucleic acid extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the method utilized.

One preferred type of sample which can be utilized in the present invention is derived from isolated neuronal tissues and cells. Such cells can be used to prepare a suitable extract or can be used in procedures based on *in situ* analysis.

The materials used in the above assay methods are ideally suited for the preparation of a kit. For example, for amplification based detection systems, the invention provides a compartmentalized kit to receive in close confinement, one or more containers which comprises:

(a) a first container comprising one or more of the amplification primers or probes of the present invention; and

(b) one or more other containers comprising one or more of the following: a sample reservoir, amplification reagents, wash reagents, and detection reagents.

In detail, a compartmentalized kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the

antibodies or probes used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or the hybridized probe.

For nucleic acid probes, examples of detection reagents include, but are not limited to radiolabeled probes, enzymatic labeled probes (horse radish peroxidase, alkaline phosphatase), and affinity labeled probes (biotin, avidin, or steptavidin). For antibodies, examples of detection reagents include, but are not limited to, labelled secondary antibodies, or in the alternative, if the primary antibody is labelled, the chromophoric, enzymatic, or antibody binding reagents which are capable of reacting with the labelled antibody. One skilled in the art will readily recognize that the antibodies and nucleic acid probes described in the present invention can readily be incorporated into one of the established kit formats which are well known in the art.

The nucleotide sequences of the present invention (SEQ ID NO:3-11, 28 and 29) are derived from a rat source. However, due to the highly conserved nature of nucleotide sequences for brain specific transcripts, the rat sequences can be used in human studies or can used to isolate the human homologues of the sequences herein disclosed. As used herein, a human homologue of a rat nucleotide sequence refers to a nucleotide sequence which contains greater than 80% homology with the described rat sequences. Such human homologues can be generated using known techniques which employ the sequences herein described as hybridization probes or amplification primers. A skilled artisan can routinely isolate the human homologues of the sequences herein described without undue experimentation.

The present invention is described in further detail in the following non-limiting examples.

EXAMPLE 1

Rat ATF

To define the expression of the GDNF gene product in normal brain cells, the highly sensitive polymerase chain reaction (PCR) was used to examine GDNF mRNA expression in acutely dissected rat SN. Regional expression was defined in the brain *in vivo*, and cell-type specific expression within the SN was determined using cultured T1 astrocytes and neurons. Tissue and T1 astrocytes were examined from several rat brain regions to define the regional and cell-specific distribution of GDNF.

GDNF-specific oligonucleotide primers based on 5′ and 3′ untranslated regions were synthesized based on the GenBank entry (accession # L15305) for the rat GDNF mRNA (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)). The expected 700 base pair (bp) fragment corresponding to the full length rat GDNF mRNA (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)) was amplified from several brain RNA sources. Aliquots (20%) of respective PCR products generated were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The source of PCR templates were genomic DNA (50 ng) isolated from postnatal day 1 (P1) rat brain, Substantia nigra Type 1 astrocytes, Basal forebrain Type 1 astrocytes, Cortical Type 1 astrocytes, substantia nigra neurons, dissected P1 substantia nigra, and dissected P1 striatum.

Tissue samples for total RNA preparation and for Type 1 (T1) astrocyte cultures were obtained from P1 rat pups (Taconic Farms, Germantown, PA). T1 astrocyte cultures were prepared from dissected P1 ventral mesencephalon containing substantia nigra (SN), basal forebrain (BF), or cortex (CTX) as previously described (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)). The dissociated neuronal cultures were established from

embryonic day 16 (E16) rat SN using previously described methods (O'Malley, E.K., *et al.*, *Exp. Neurol. 112*:40-48 (1991); O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)). Total RNA was isolated by lysing either T1 astrocyte monolayers, dissociated neuronal cultures, or dissected tissues in 4M guanidinium isothiocyanate, 25mM sodium citrate (pH 7.0), 0.1M β-mercaptoethanol, followed by cesium chloride centrifugation (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). First strand cDNA was synthesized from total RNA (5μg) using Superscript RNAse H$^-$ reverse transcriptase (RT) (Gibco/BRL, Gaithersburg, MD) primed by 200 ng random hexamers (Promega, Madison, WI) as per manufacturer's instructions. The first strand reaction was terminated by heating at 80°C for 10 minutes, and an aliquot (12.5%) of the RT reaction was utilized as template in the PCR. The RT-PCR was performed by standard methodologies (Kawasaki, E.S., "Amplification of RNA," in *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, *et al.*, eds.), pp. 21-27, Academic Press, San Diego (1990)). Briefly, reactions consisted of: 50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 0.2mM dNTP's, 0.2μM GDNF primers*, and 1.0mM $MgCl_2$. Reactions were overlaid with mineral oil, initially denatured at 95°C for 7 minutes and 2.5U Taq DNA polymerase (Promega) was added. Thermocycling parameters were: 1 minute denaturation at 95°C; a 30 second ramp to 60°C; 1 minute annealing at 60°C; a 30 second ramp to 72°C; 1 minute primer extension at 72°C; repeated for 30 cycles. Sense primer was (5′)GGTCTACGGAGACCGGATCCGAGGTGC (3′) (SEQ ID NO:12) and the anti-sense was (5′)TCTCTGGAGCCAGGGTCAGATACATC (3′) (SEQ ID NO:13) corresponding to bases 1-27 and 675-700 respectively in the GDNF mRNA (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)).

The PCR product generated from SN T1 astrocyte mRNA was cloned and sequenced using the Sanger dideoxy termination method (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA. 74*:5463-5467 (1977)). The sequencing results confirmed that the 700 bp PCR product was identical to the published rat GDNF sequence (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)). In addition, a smaller molecular weight PCR product, evident in the striatum and in SN and BF Type 1 astrocytes, was consistently co-amplified with the full-length GDNF cDNA. Sequence analysis of the smaller PCR product showed it to be an alternative form of GDNF, identical to the previously reported nucleotide sequence with the exception of a deletion encompassing 78 bp. This smaller species has been named ATF-1 and the nucleotide amino acid sequence are disclosed in SEQ ID NO:1 and 2.

Human ATF

Human RNA was obtained from Jim Krause (Washington Univ. School of Medicine, Washington Univ. Medical Center, Department of Anatomy and Neurobiology, St. Louis, MO) (for RNA isolation techniques see: Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). All RNA was obtained from patients autopsied. The tissue was removed 1 to 6 hours post mortum. No neurological disease was apparent. Human caudate and putamen RNA used in this study were isolated from the same patient (patient #3).

PCR products were generated from human RNA as described in Schaar *et al.*, *Exper. Neurol. 124*:368-371 (1993). Briefly, first strand cDNA was synthesized from total RNA (approximately 2.5 μg) using Superscript RNAse H^- reverse transcriptase (RT) (Gibco/BRL, Gaithersburg, MD) primed by 200

ng random hexamers (Promega, Madison, WI) as per manufacturer's instructions. The first strand reaction was terminated by heating at 80°C for 10 minutes, and an aliquot (approximately 10%) of the RT reaction was utilized as template in the PCR. The RT-PCR was performed by standard methodologies (Kawasaki, E.S., "Amplification of RNA," in *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, *et al.*, eds.), pp. 21-27, Academic Press, San Diego (1990)). Briefly, reactions consisted of: 50mM KCl, 10mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 0.2mM dNTP's, 0.2μM GDNF primers*, and 1.0mM $MgCl_2$. Reactions were overlaid with mineral oil, initially denatured at 95°C for 7 minutes and 2.5U Taq DNA polymerase (Promega) was added. Thermocycling parameters were: 1 minute denaturation at 95°C; a 30 second ramp to 60°C; 1 minute annealing at 60°C; a 30 second ramp to 72°C; 1 minute primer extension at 72°C; repeated for 35 cycles. Sense primer was (5′)GGTCTACGGAGACCGGATCCGAGGTGC (3′) (SEQ ID NO:12) and the anti-sense was (5′)TCTCTGGAGCCAGGGTCAGATACATC (3′) (SEQ ID NO:13) corresponding to bases 1-27 and 675-700 respectively in the GDNF mRNA (Lin, L.F.H., *et al.*, *Science 260*:1130-1132 (1993)).

The PCR product generated from human caudate RNA was subcloned using the TA Cloning System (In Vitrogen). Six clones were sequenced using the Sanger dideoxy termination method (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA. 74*:5463-5467 (1977)). Clones #3 and #17 corresponded to human GDNF (SEQ ID NO:36 and 37, nucleotide and amino acid sequence, respectively). Clones #5 and #16 were identical and termed ATF-1 (SEQ ID NO:30 and 31, nucleotide and amino acid sequence, respectively). Clone #18 was termed ATF-2 (SEQ ID NO:32 and 33, nucleotide and amino acid sequence, respectively).

Rat ATF-3 Isolation and Analysis of ATF RNA Expression

Tissue samples were dissected from E17, P1 and P32 rats, previously anaesthetized on dry ice. Total RNA was isolated using the method of Chomczynski, P. and Sacchi, N., *Anal. Biochem. 162:*156-159 (1987). Reverse transcription and PCR were essentially as described by Schaar, D.G. *et al., Expt. Neurol. 124:*368-371 (1993). The two changes were to increase the annealing temperature to 65°C and to use newly designed, shorter 5′ and 3′ primers. The sequence of the primers was GTCTACGGAGACCGGATCCG and CTCTGGAGCCAGGGTCAGATAC (5′ (SEQ ID NO:38) and 3′ (SEQ ID NO:39), respectively) They correspond to bases 2-21 and 678-699 of the rat GDNF sequence. The numbering is according to the rat GDNF sequence in the Genbank database (Accession # L15305).

Using RT-PCR, the developmental and regional expression of GDNF and its alternate transcripts in rat brain was studied. The tissues implicated in the nigro-striatal pathway were examined. mRNA levels in the substantia nigra (SN) declined from embryonic day 17 (E17) to postnatal day 32 (P32), while levels in the striatum remained constant. The RT-PCR also revealed three GDNF-related transcripts in SN and striatum; GDNF, ATF-1 and ATF-3. ATF-1 has a 78 bp deletion in the GDNF prepro region. Sequence analysis of ATF-3 revealed it has a 177 bp deletion in the GDNF prepro region (ATF-3 amino acid and nucleotide sequence: SEQ ID NO:36 AND SEQ ID NO:37, respectively). This deletion begins with nucleotide 24 and ends with nucleotide 200. The excision removes both the proposed start codon for the gene and the signal sequence for secretion.

The expression pattern in cerebellum, cortex, hippocampus and septum was examined. The results showed mRNA levels in these four tissues declined

from P1 to P32. Prior to this timepoint, there was regional specificity in expression. In cortex and septum, the message levels increased from E17 to P1; in hippocampus they remained constant from E17 to P1 and in cerebellum they rapidly decreased from E17 to P1. The three transcripts of GDNF were present in each of these tissues.

Rat ATF Expression

Results of the PCR analysis indicate that GDNF is expressed prominently in cultured SN T1 astrocytes. However, in freshly dissected SN, levels of GDNF mRNA were at the threshold of detectability, in some gels, no band was detected. Levels of GDNF mRNA in whole SN tissue were lower than in SN T1 astrocytes in several experiments. Thus, the primary cell culturing process may up-regulate GDNF mRNA expression. Similar results have been reported with NGF expression in rat optic nerve (Lu, B., *et al.*, *Exp. Neurol. 104*:191-199 (1989)). Although barely detectable in the postnatal optic nerve, NGF mRNA expression is increased in primary cell culture of optic nerve astrocytes. GDNF mRNA expression in the SN may be similarly regulated.

SN neuronal dissociates also express GDNF at low but detectable levels. Neuronal expression may reflect a low level of glial contamination in the neuronal cultures (<3%). Alternatively, GDNF may be expressed by neurons. In aggregate, these observations suggest that SN T1 astrocytes, and possibly neurons, are a source of GDNF. Since GDNF enhances survival of DA cells, these data support a local role for trophic factors, consistent with previous studies (Korsching, S., *J. Neurosci. 13*:2739-2748 (1993); Lu, B., *et al.*, *Exp. Neurol. 104*:191-199 (1989)).

In addition to locally-acting molecules, target-derived factors play an important role in maintaining neuronal survival. Several studies have suggested that striatal glial and factors contribute to the survival and differentiation of DA neurons *in vitro* (Appel and Tomozawa, *Brain Res.* *399*:111-124 (1986); Dal Toso, R., *et al.*, *J. Neurosci.* *8*:733-745 (1988); Rousselet, A., *et al.*, *Dev. Biol.* *129*:495-504 (1988)). To determine whether GDNF could be a target-derived factor for nigral DA neurons, rat striatum was examined *in vivo*. High levels of GDNF message were observed in freshly dissected P1 striatum, suggesting that target expression is important for survival of nigrostriatal DA neurons.

To determine whether the expression of GDNF is widespread, cortex and basal forebrain, two areas that degenerate in Alzheimer's disease were examined (Davies and Maloney, *Lancet* 2:1403 (1976); Whitehouse, P.J., *et al.*, *Ann. Neurol.* *10*:122-126 (1981)). Cortical astrocytes expressed low levels of GDNF, while basal forebrain astrocytes expressed more moderate GDNF mRNA levels. These observations suggest that the actions of this factor may extend to neuronal populations other than DA neurons.

The results indicate that GDNF is expressed in the nigrostriatal system, supporting the proposal that GDNF is a survival factor for nigrostriatal DA neurons. In addition to GDNF, several previously identified trophic agents reportedly enhance the survival of DA neurons in mesencephalic cultures. These include basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and brain-derived neurotrophic factor (BDNF) (Beck, K.D., *et al.*, *Neuroscience* *52*:855-866 (1993); Casper, D., *et al.*, *J. Neurosci. Res.* *30*:372-381 (1991); Engele and Bohn, *J. Neurosci.* *11*:3070-3078 (1991); Ferrari, G., *et al.*, *Dev. Biol.* *133*:140-147 (1989); Hyman, C., *et al.*, *Nature* *350*:339-341 (1990)). Moreover, BDNF (Beck, K.D., *et al.*,

Neurodegeneration 1:27-36 (1992); Hyman, C., *et al.*, *Nature 350*:339-341 (1990); Skaper, S.D., *et al.*, *J. Neurosci. Res. 34*:478-487 (1993); Spina, M.B., *et al.*, *J. Neurochem. 59*:99-106 (1992)) and bFGF (Park and Mytilineou, *Brain Res. 599*:83-97 (1992)) have been shown to protect against neurotoxic insult in an *in vitro* model of Parkinson's disease. In contrast to GDNF, none of these factors appears selective for DA neurons *in vitro*. Both EGF and bFGF are mitogenic for glial cells, which mediate their trophic actions on DA neurons (Casper, D., *et al.*, *J. Neurosci. Res. 30*:372-381 (1991); Engele and Bohn, *J. Neurosci. 11*:3070-3078 (1991); Park and Mytilineou, *Brain Res. 599*:83-97 (1992)). BDNF also affects GABA neurons within the SN (Hyman, C., *et al.*, *Nature 350*:339-341 (1990); Skaper, S.D., *et al.*, *J. Neurosci. Res. 34*:478-487 (1993)).

Despite the selective effects of GDNF on SN DA neurons, GDNF expression in cortical and basal forebrain T1 astrocytes suggests a more generalized role in the central nervous system. Such actions have been described for bFGF and BDNF. bFGF increases neuronal survival in cortex (Morrison, R.S., *et al.*, *Proc. Natl. Acad. Sci. USA 83*:7537-7541 (1986)) and hippocampus (Walicke, P., *et al.*, *Proc. Natl. Acad. Sci. USA 83*:3012-3016 (1986)). Survival of basal forebrain cholinergic neurons (Alderson, R.F., *et al.*, *Neuron 5*:297-306 (1990); Friedman, W.J., *et al.*, *Exp. Neurol. 119*:72-78 (1993)) and retinal ganglion cells (Johnson, J.E., *et al.*, *J. Neurosci. 6*:3031-3038 (1986)) is increased by BDNF. These factors, like GDNF, are expressed in neurons as well as in glial cells (Gaul and Lubbert, *Proc. Roy. Soc. London. Ser. B. 249*:57-63 (1992); Pettman, G.M., *et al.*, *Neurosci. Lett. 68*:175-180 (1986)). Results suggest that GDNF, which is a selective dopaminergic factor in cultures of ventral mesencephalon, may also enhance neuronal survival in other brain areas.

The high levels of GDNF expression in the nigrostriatal system suggest that the molecule regulates survival of SN DA neurons *in vivo*. In separate studies, a factor elaborated by local T1 astrocytes selectively enhanced the survival of SN DA neurons *in vitro* (O'Malley, E.K., *et al.*, *Society for Neuroscience Abstracts 18*:1292 (1992)). The localization of GDNF to SN T1 astrocytes raises the possibility that this astrocytic factor is similar or identical to GDNF.

To determine whether GDNF and ATF may have similar biological actions, basal forebrain cholinergic neurons, substantia nigra dopamine neurons and locus coeruleus norepinephrine neurons were evaluated in dissociated cultures. Brain regions were dissected from embryonic day 16 rats, dissociated, and cultures were exposed to the putative trophic molecules for 7 days. GDNF and ATF were generated in COS cells transfected with the appropriate plasmids using established techniques (Felgner et al, *Proc. Natl. Acad. Sci. 84*:7413-7417 (1987). Mock transfected COS cell medium served as control. COS cell medium was concentrated approximately 20-fold using a Amicon Centriprep 10 with a cut-off 10kD. In all cases 25 μl of the COS cell concentrate was added to 1 ml serum free medium.

Human ATF Expression

The PCR product obtained from human SN, Caudate, and Putamen RNA using the primers described above was analyzed by agarose gel electrophoresis. Approximately 15% of the PCR reactions were loaded and electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and photographed (Figure 5). Lane 1 contains lambda BstE II molecular weight markers. Lane 2 contains the product of a mock PCR reaction with reverse transcriptase but without RNA template. Lane 3

contains the product of a PCR reaction with 50 ng human brain genomic DNA. Lane 4 contains the product of a PCR reaction using RNA isolated from Rat cultured type 1 astrocytes. Here, GDNF amplification was greater than ATF amplification. Lane 5 contains the product of a PCR reaction using isolated human SN RNA. No amplification was observed using human SN RNA. Lane 6 contains the product of a PCR reaction using human caudate RNA Here, ATF amplification was greater than GDNF amplification. Lane 7 contains the product of a PCR reaction using human putamen RNA. Here, low levels of GDNF and ATF were amplified.

Human ATF-1 and ATF-2 Transcript Analysis

PCR analysis was conducted utilizing primers which bridge the GDNF sequences deleted in ATF-1 and ATF-2. These bridging primers were designed to specifically amplify ATF-1 or ATF-2 mRNA; full-length GDNF mRNA cannot be amplified using these primer sets. The 5′ ATF-1 bridging primer consisted of human GDNF bases 108-122 and 201-217 (5′ ^{108}TCCCGCTGCCCGCCG^{122} ^{201}CAAATATGCCAGAGGAT^{217} 3′, SEQ ID NO:40) which spans the 78 base pair GDNF region deleted in ATF-1. Similarly, the 5′ ATF-2 bridging primer consisted of human GDNF bases 95-111 and 338-353 (5′ ^{95}ACCGCGTCCGCCTTCCC^{111} ^{338}GCTGCCAACCCAGAGA^{353} 3′, SEQ ID NO:41) which spans the GDNF region not present in the ATF-2 cDNA. The 3′ PCR primer employed in these experiments corresponded to the 3′ GDNF untranslated region (bases 675-700) (Schaar, D.G. *et al., Exp. Neurol.* 124:368-371 (1993)). The PCR methodology and RNA sources were as described in Schaar, D.G. *et al., Exp. Neurol.* 124:368-371 (1993) and herein. After 35 cycles of

amplification, an aliquot from each reaction was analyzed by agarose gel electrophoresis with visualization by ethidium bromide staining.

The ATF-1 bridging PCR resulted in the amplification of a 515 base pair product as would be expected from the primers utilized and the existence of the appropriate mRNA template. Also the ATF-2 bridging PCR amplified a 380 base pair product verifying the existence of a processed ATF-2 RNA template. Thus both ATF-1 and ATF-2 are expressed at the RNA level in the human adult brain. In addition, ATF-1 and ATF-2 RNA's are expressed, at varying levels, in the three human brain regions analyzed (substantia nigra, caudate and putamen).

GDNF Protein Processing

GDNF protein processing was analyzed through pulse-chase experiments employing lysates of Sf9 cells that had been infected with a recombinant baculovirus containing the rat GDNF cDNA. The pulse-chase procedure was adapted from the method of Jarvis and Summers, *Mol. Cell Bio. 9:*214-223 (1989); construction of the GDNF baculovirus was performed essentially as per manufacturer's instructions (Pharmingen, San Diego, CA). Briefly, the Sf9 cells were grown in IPL-41 medium (Gibco-BRL, Gaithersburg, MD) with 5% fetal calf serum (FCS), in suspension at a density of 2 X 10^6 cells/ml and inoculated with a multiplicity of infection (MOI) of 50 pfu/cell. At 20 hours post-infection, the cells were harvested, resupended in 10 ml Methionine-free medium (Gibco-BRL) and starved for 60 minutes at 27^0C. Following the starvation period, the cells were pulse-labeled for 5 minutes with 500μCi/ml of translabel-^{35}S (EXPRE ^{35}S ^{35}S, NEN, Wilmington, DE). The cells were then diluted 1:10 in chase medium (IPL-41 + 5% FCS), centrifuged and resuspended in a final volume of 100 mls. The

zero-time point samples were taken at this time. Samples were removed at the following time points: 5, 10, 15, 30, 45, 60, 90 and 120 minutes. The cells from each time point were centrifuged and lysed in 1ml RIPA buffer (0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15M NaCl, 0.01M Tris-HCl pH 7.4, 0.001M EDTA, 0.2mM PMSF). Immunoprecipitations were performed on the cell lysates employing an anti-GDNF rabbit polyclonal antibody conjugated to agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); 5μl anti-GDNF beads (500μg IgG/250μl agarose) were used per ml lysate. After 48 hours at 4°C, immunoprecipitates were collected, washed once in RIPA and disrupted by boiling in 30μl of protein sample buffer for 2 minutes. Samples were analyzed on a 12.5% SDS-PAGE, which was subsequently dried and exposed to x-ray film.

The order of appearance of the three immunoreactive polypeptides (approximate molecular mass 23, 28, 33 kd) which were consistently seen on western blots of GDNF transfected, Sf9 lysates were determined in a pulse chase experiment. Two bands of molecular masses 28 and 33 kd were visualized on the autoradiogram in equal amounts throughout the 120 minute chase period. The 23 kd band did not appear on this autoradiogram. A labeled protein of apparent molecular weight 40 kd did increase in intensity during the chase period. These findings are consistent with the 28 and 33 kd bands being translated from separate RNAs. The 23 kd band may be less efficiently translated or less stable, may not contain methionine or cysteine residues, or may be a degradation product only seen at later times. The 40 kd protein has never been visualized on western blots suggesting that it co-precipitates with GDNF. The progressive increase in the high molecular weight 40 kd band raises the possibility that it represents a post-translationally processed form of the low molecular weight species.

EXAMPLE 2

Materials and Methods:

Cell Culture and RNA Isolation

Dissociated cell cultures were established from rat P1 substantia nigra, fractionated into cultures enriched for either type 1 of type 2 astrocytes by the method of McCarthy and DeVellis (McCarthy and DeVellis, *J. Cell Biol. 85*:890-902 (1980)), with modifications described by O'Malley *et al.* (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)). Glial fractionation procedures generated type 1 astrocyte cultures containing >95% GFAP positive cells, and type 2 cultures consisting of approximately 70% A2B5 immunoreactive cells (O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)). In the dissection of the mesencephalon, tissue ventral to the cerebral aqueduct (containing the SN) was referred to as ventral mesencephalon, the remaining tissue was designated dorsal mesencephalon. Total RNA was isolated by lysing glial monolayers and dissected tissues in 4 M guanidium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, followed by cesium chloride centrifugation (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

First Strand cDNA Synthesis

First strand cDNA was synthesized from 5 μg astrocyte total RNA using Superscript RNAse H^- reverse transcriptase (Gibco/BRL, Gaithersburg, MD) primed by a Not-dT oligo [$CCGGCTCGAGCGGCCGC(T)_{18}$] SEQ ID NO:14, as per manufacturer's instructions. The reverse transcription was terminated by heating reactions at 80°C for 10 minutes; excess first strand

primers were removed by ion exchange chromatography on Qiagen-5 columns (Qiagen, Chatsworth, CA). First strand products were eluted with 1.25 M NaCl, 50 mM MOPS (pH 8.2), 15% ethanol; adjusted to 20 μg/ml linear polyacrylamide and ethanol precipitated. The cDNA was resuspended in sterile water, extracted once with an equal volume of phenol, followed by two chloroform/isoamyl alcohol (24:1) (CIA) extractions and re-precipitated with ethanol. The astrocyte cDNA was collected by centrifugation, washed twice with 70% ethanol, then dried *in vacuo*.

An aliquot (25%) of the first strand products was resuspended in 19 μl terminal deoxynucleotidyl transferase (TdT) buffer (5X TdT buffer is: 500 mM cacodylate buffer (pH 6.8); 5 mM $CoCl_2$; 0.5 mM dithiothreitol; 500 μg/ml BSA). A dG tail was added to the 3' cDNA terminus by adding dGTP (Pharmacia, Piscataway, NJ) to a final concentration of 0.165 μM, incubating at 37°C with 23 units of terminal deoxynucleotidyl transferase (Promega, Madison, WI) for 20 minutes. Tailing reactions were terminated by adding NaCl to 0.125 M and EDTA to a final concentration of 12.5 mM. The RNA was hydrolyzed at this point by adjusting reactions to 50 mM NaOH and heating at 70°C for one hour. Reactions were neutralized by adding 0.1 volumes of 3 M sodium acetate (pH 5.2), and ethanol precipitated.

Non-Sequence Specific cDNA Amplification

Tailed first strand cDNA was utilized as template in a non-sequence specific cDNA amplification, as described previously by Belyavsky *et al.* (Belyavsky, A., *Nuc. Acids Res. 17*:2919-2932 (1989)) and also Brunet *et al.* (Brunet, J.F., *et al.*, *Science 252*:856-859 (1991)) with few modifications. The polymerase chain reactions consisted of: 10 μl dG tailed first strand (corresponding to the reverse transcription of approximately 1 μg total RNA);

0.2 mM dNTP's (Pharmacia, Piscataway, NJ); 50 mM KCl; 10 mM Tris-HCl (pH 9.0 at 25°C); 1.5 mM $MgCl_2$; 0.01% gelatin (w/v); 0.1% Triton X-100; 0.2 μM Not-dT and Not-dC (CCGGCTCGAGCGGCCGCC$(C)_{12}$) (SEQ ID NO:15) oligo's; 5 units Taq polymerase (Promega, Madison, WI); reactions overlaid with mineral oil prior to thermocycling. The PCR conditions for the initial 5 cycles were denaturation at 95°C for 2 minutes; a 1 minute 30 second ramp to 37°C; 2 minute annealing at 37°C; a 1 minute ramp to 72°C; primer extension at 72°C for 4 minutes. The next twenty cycles were performed under the same conditions, however the annealing temperature was increased to 50°C. The final cycle was a 10 minute primer extension at 72°C to ensure the completion of double-stranded products.

The amplified cDNA was extracted with phenol, followed by two CIA extractions, ethanol precipitated and collected by centrifugation. PCR products were resuspended in Not 1 restriction buffer and digested with 15 units Not 1 (Promega, Madison, WI). After digestion, the cDNA was loaded on a 2% NuSieve (FMC, Rockland, ME); cDNA that ranged from 200 to 1000 base pairs was recovered from the gel. At this point, the Not 1 digested cDNA was either ligated into the pGEM-5zf(+) sequencing vector (Promega, Madison, WI), or utilized as template for asymmetric PCR for subsequent subtractive cloning methodologies.

Asymmetric PCR

Asymmetric PCR's were employed to generate single-stranded cDNA as described by McCabe (McCabe, P.C., "Production of single-stranded DNA by asymmetric PCR," in *PCR Protocols: A Guide to Methods and Applications,* Innis *et al.*, (eds.), Academic Press, San Diego, CA, pp. 76-83 (1990)) with modifications for application in subtractive cloning

methodologies. Briefly, reactions consisted of: 50μM dNTP's (in type 2 asymmetric PCRs, dUTP was substituted for dTTP; the type 1 PCRs were radiolabeled by including 10 μCi alpha-^{32}P-dCTP in the reaction mix); 50 pmol excess primer; 1pmol limiting primer; 50 mM KCl; 10 mM Tris-HCl (pH 9.0 at 25°C); 1.5 mM $MgCl_2$; 0.01% gelatin; 0.1% Triton X-100; 2.5 units Taq polymerase (Promega, Madison, WI). PCR conditions included an initial denaturation at 95°C for 12 minutes, then 30 cycles as follows: 30 second denaturation at 95°C; a one minute 30 second slope down to 60°C; 30 second annealing at 60°C; a one minute ramp up to 72°C; 2 minute primer extension at 72°C. The final cycle was a 10 minute incubation at 72°C to complete remaining primer extensions. The resultant asymmetric PCR products were precipitated by adding a one-half volume 7.5 M ammonium acetate, 2.5 volumes ethanol and storing at -20°C until use. Asymmetric PCRs were analyzed on a 2% NuSieve (FMC) agarose gel to check size distribution and estimate DNA concentrations from ethidium bromide staining, typically 4-5 μg PCR products were synthesized per reaction.

Subtractive Hybridization

The rationale underlying the selection of hybridization parameters has been recently summarized (Calzone, F.J., *et al.*, "Mapping of gene transcripts by nuclease protection assays and cDNA primer extension," in Berger and Kimmel (eds.), *Methods in Enzymology: Guide to Molecular Cloning Techniques*, Vol. 152, pp. 611-632, Academic Press, San Diego, CA (1987)). Typically, a 20-40 fold excess of type 2 sense cDNA was co-precipitated with type 1 antisense cDNA. The cDNA mixture was resuspended in: 50% formamide; 0.4 M NaCl; 1 mM EDTA; 20 mM PIPES (pH 6.5); in a 10 μl final volume. The reaction was overlaid with mineral oil and allowed to

hybridize at 46.6°C for 12-16 hours. After hybridization, the aqueous phase was recovered by extracting twice with CIA, then added directly to 200 μl of 0.2 mg/ml sonicated salmon sperm DNA in 50 mM $NaPO_4$ (pH 7.0). The single-stranded cDNA, corresponding to type 1 specific cDNA as well as excess type 2 subtracting sequences, was recovered through batch hydroxylapatite (HAP) (Bio-Rad, Richmond, CA) chromatography as described by Jackson and Felsenfeld (Jackson and Felsenfeld, "*In vivo* Footprinting of Specific Protein-DNA Interactions," in *Methods in Enzymology: Guide to Molecular Cloning Techniques, Vol. 152,* pp. 735-755, S.L. Berger and A.R. Kimmel (eds.), Academic Press, San Diego, CA (1987)). The eluted single-stranded fraction was cleared of residual HAP by filtering through a 0.22 μM Millex-GV filter unit (Millipore, Bedford, MA), then subjected to three rounds of desalting and concentration by dilution in TE (10 mM Tris-HCl pH 7.5; 1 mM EDTA) and centrifugation in Centricon-30 concentrators (Amicon, Beverly, MA). The collected cDNA was ethanol precipitated in the presence of linear acrylamide carrier as described previously.

The eluted single-stranded cDNA was resuspended in glycosylase buffer (10X glycosylase buffer is 600 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM DTT, 1 mg/ml BSA) and incubated with 2 units uracil DNA glycosylase (Boehringer Mannheim, W. Germany) at 37°C for one hour. After the glycosylase treatment, 16.5 μl 0.6 N NaOH was added to reactions and the 37°C incubation was continued for 5 minutes to hydrolyze apyrimidinic sites. The reaction was neutralized with 16.5 μl 0.6 N HCl, remaining single-stranded cDNA was ethanol precipitated with carrier then resuspended in TE, CIA extracted and re-precipitated.

Type 1 Astrocyte-Specific cDNA Cloning

The subtracted, type 1-specific first strand cDNA was subjected to a limited PCR amplification to complete the double-stranded DNA structure and to facilitate cloning into an appropriate sequencing vector. PCR amplification of the subtracted template was performed as described under non-sequence-specific PCR. However, we employed a shorter primer extension step (two minutes) and the PCR was limited to 15 cycles. The PCR products generated were CIA extracted; precipitated by adding a one-half volume 7.5 M ammonium acetate and 2.5 volumes ethanol. This precipitate was collected by centrifugation, resuspended in Not 1 restriction buffer and digested exhaustively with Not 1 (Promega, Madison, WI) at 37°C overnight. The digested products were separated on a 2% NuSieve agarose gel, DNA ranging in size from 200 to 1000 base pairs was recovered from the gel, ligated into Not 1 restricted pGEM-5zf(+) and used to transform competent DH5 alpha cells (Gibco/BRL). The type 1 subtracted library contained 2.4 X 10^3 recombinants which were replica plated on Hybond-N+ membranes (Amersham Life Sciences, Arlington, Heights, IL) and screened by colony hybridization utilizing first strand cDNA probes generated from type 1 and type 2 astrocyte RNA, as described by Sargent (Sargent, T.D., "Isolation of differentially expressed genes," in *Methods in Enzymology: Guide to Molecular Cloning Techniques, Vol 152,* Berger and Kimmel (eds.), Academic Press, San Diego, CA, pp. 423-432 (1987)).

Northern Analysis

Northern blotting was performed using nylon membranes (Gene Screen, NEN Research Products, Boston, MA) or supported nitrocellulose (Hybond C Super, Amersham) following manufacturer's specifications. Blots

were prehybridized in: 50% formamide, 10% dextran sulphate, 1 M NaCl, 1% SDS at 42°C for 1-2 hours. Hybridizations were performed in the same solution, adding random-primed probes (Feinberg and Vogelstein, *Analytical Biochem. 132*:6-13 (1983)) to a concentration of 0.5 -1.0 X 10^6 cpm per ml hybridization fluid and incubating overnight at 42°C. Blots were washed under stringent conditions (0.1 X SSC; 0.1% SDS at 65°C) and autoradiographed for varying exposures at -80°C with intensifying screens.

DNA Sequencing

The astrocyte type 1-subtracted clones were sequenced using the dideoxy termination method originally described by Sanger *et al.* (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 74*:5463-5467 (1977)). Chain-termination sequencing was performed utilizing a modified T7 DNA polymerase (Sequenase, United States Biochemical, Cleveland, Ohio) and also Taq DNA polymerase (Gibco/BRL) as per manufacturer's instructions. Acquired type 1 sequences were analyzed using the Genetics Computer Group (GCG) Sequence Analysis Software Package (GCG, Inc., Madison, WI).

in situ Hybridization

All *in situ* hybridizations were performed utilizing ^{35}S-labelled riboprobes. The transcription mixture consisted of 40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 40 units RNasin (Promega), 100 ug/ml BSA, 0.4 mM each of ATP, GTP, UTP, 0.08 mM CTP, approximately 17 uM ^{35}S-CTP, 1 ug DNA template, 10 units RNA (T7 or Sp6) polymerase (Promega). Reactions were incubated at 37°C for one hour, then phenol extracted and purified by column chromatography on CF 11 resin. The length of the probe was verified by polyacrylamide gel electrophoresis followed by X-ray film

autoradiography. The *in situ* hybridization procedure was performed essentially as described by Hogan *et al.* (Hogan, B., *et al.*, *Manipulating the Mouse Embryo: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1986)).

Results:

Construction of a Type 1 Astrocyte Subtracted Library

The PCR-based subtractive cloning methodology (outlined schematically in Figure 1) resulted in the construction of a type 1-specific library from trace amounts of mRNA isolated from highly enriched primary glial cultures. Rat P1 substantia nigra dissociates were fractionated into glial subtype cultures, enriched for either type 1 (>95% cells GFAP positive) or type 2 astrocytes (approximately 70% cells A2B5 positive). One microgram astrocyte total RNA (corresponding to approximately 10-50 ng mRNA), was utilized to generate a library consisting of 2400 recombinants. An initial colony hybridization screen utilizing radiolabeled first strand cDNA probes specific to each glial subtype yielded 200-300 clones which hybridized exclusively or differentially with type 1 astrocyte probes. The second round of screening identified twenty-one clones which maintained this type 1 specificity. These twenty-one clones were classified by cDNA insert size; recombinants containing equivalent insert sizes were provisionally presumed to be identical, pending sequence determination.

Partial Sequence Analysis of Astrocyte Type 1 (AT1) Clones

Partial sequence analysis was performed on 11 of the type 1-specific clones (see table 1 for a summary of AT1 sequence analysis). Three AT1 clones represented previously identified genes. Two sequence matches were

not informative as AT1-48 and AT1-21 were shown to be partial cDNA clones of 18s rRNA and 28s rRNA respectively. In contrast, AT1-40 was identified as a partial cDNA corresponding to a rat peroxisomal membrane protein (PMP70) (Kamijo, K., *et al.*, *J. Biol. Chem. 265*:4534-4540 (1990)), one of the major peroxisome-specific proteins, which presumably plays a role in formation of the organelle. Peroxisomes contain numerous enzymes which catalyze many metabolic functions, including the β-oxidation of very long chain fatty acids (van den Bosch, H., *et al.*, *Ann. Rev. Biochem. 61*:157-197 (1992)).

A group of five AT1 cDNA's contain rat-specific repetitive sequence insertions ranging from 36-85 nucleotides in length. These mobile repetitive elements have been termed short interspersed repeated sequences (SINES), and represent nonviral retroposons (for review see Weiner *et al.* (Weiner, A.M., *et al.*, *Ann. Rev. Biochem. 55*:631-661 (1986))). The AT1 cDNA sequences 5' to these repetitive elements do not share significant homologies with existing database entries (Genebank/EMBL), raising the possibility that the repetitive elements have been inserted into novel, astrocyte-specific genes.

Three remaining AT1 cDNA's (46, 911, and 45 (two fragments) SEQ ID NO:3, 4, 28 and 29, respectively) contained inserts ranging from 250-830 bp, and subsequent partial sequence determination (100-200 bp in from each cDNA end) enabled database searches to examine possible identities with previously reported genes. In fact, AT1 cDNA's 46, 911, and 45 represent novel astrocytic genes: they do not share significant homologies with existing database sequences, and also do not contain obvious stretches of repetitive sequence. These cDNA's represent previously undescribed genes differentially expressed by type 1 astrocytes.

Regional Expression of AT1-40

AT1-40 was chosen to demonstrate how the sequences of the present invention can be used to assay for astrocyte specific expression. Astrocyte type 1 clone #40 (AT1-40) corresponds to the 3' untranslated region (base 2443-2820) of the rat peroxisomal membrane protein (PMP70) (Kamijo, K., *et al.*, *J. Biol. Chem. 265*:4534-4540 (1990)). Peroxisomes are organelles present in nearly all eukaryotic cells, number and size varying with physiologic state and cell type (Deduve and Baudhuin, *Physiol. Rev. 46*:323-357 (1966)). PMP70 (AT1-40) is a major component of the peroxisome membrane and also is an ATP-binding cassette protein (Kamijo, K., *et al.*, *J. Biol. Chem. 265*:4534-4540 (1990)). Mutations in this protein have been identified in the cerebro-hepato-renal, Zellweger Syndrome (ZS), characterized by defective peroxisome biogenesis, implying a role for PMP70 in the formation of these organelles (Gärtner, J., *et al.*, *Nature Genetics 1*:16-23 (1992)). To examine the regional and potential functional specificity of PMP70, the level of AT1-40 (PMP70) gene expression was examined in specific glial subtypes (astrocyte type 1 versus 2) in different brain regions.

As expected, AT1-40 RNA expression was detected in all brain regions examined, and in multiple organ systems in the P1 animal. Elevated expression of AT1-40 in brain type 1 astrocytes, which are known to be components of the blood-brain barrier (Janzer and Raff, *Nature 325*:253-257 (1987); Raff, M.C., *Science 243*:1450-1455 (1989)), raises the possibility of function in detoxification and secretion, consistent with the known high expression of PMP70 in liver and kidney.

Although widespread, AT1-40 RNA was expressed at different levels in different brain regions. SN type 1 astrocytes expressed at least ten-fold

greater levels of AT1-40 RNA than those derived from the cortex or basal forebrain, implying region-specific differences in type 1 astrocytic function.

Characterization of a Novel Type 1 Astrocyte cDNA

Astrocyte type 1 clone # 46 (AT1-46) was chosen for further analysis. The original subtracted PCR clone contained a 831 base pair cDNA insert which did not share any significant homologies with current database entries. In Southern analysis, the AT1-46 cDNA hybridized with a single fragment of P1 brain DNA digested with multiple restriction enzymes, implying recognition of a single-copy gene. Moreover, AT1-46 recognized a low abundance 4.8 kb transcript expressed in a limited fashion in the P1 animal, particularly in the brain. The current AT1-46 RNA expression data is summarized below. The highest levels of expression were observed in the P1 kidney, and in type 1 astrocytes derived from the P1 basal forebrain. Additional experiments detected very low levels of the AT1-46 transcript in SN type 1 astrocytes and P1 lung. To more precisely map brain regions expressing AT1-46, *in situ* hybridization studies in the P1 rat brain was performed. Highest expression occurred in the olfactory neuroepithelium, cingulate, frontal and parietal cortices, caudate putamen and septum, and in the hippocampus at lower levels. These studies of AT1-46 highlight the utility of the PCR-subtraction cloning methodology to identify novel astrocytic genes that are expressed in a regionally-specific manner, and to detect differential expression of widespread gene products, such as AT1-40 (PMP70).

Expression of AT1-46 in adult rat brain was further explored using *in situ* hybridization. Dark field photomicrograph of coronal sections showed labelling in the tenia tecta (TT), rostral piriform (PC), frontal (FC) and

cingulate (CG) cortices; in caudal piriform cortex; in hippocampus (H) and dentate gyrus (DG).

High magnification, bright-field picture of cortical cells showed some cells with neuronal morphology expressing AT1-46. Some cells with neuronal morphology and darkly stained small cells with glial morphology exhibit labelling at background level.

High-magnification, bright field picture of cells in piriform cortex showed lightly stained oval cells with darker morphology which did not express AT1-46, granule cells of the dentate gyrus which expressed AT1-46 and unlabelled granule cells and cells with glial morphology that do not express AT1-46.

Discussion

Increasing evidence suggests that glial function in a regionally-specific manner in the brain (O'Malley, E.K., *et al.*, *Exp. Neurol. 112*:40-48 (1991); O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992); Prochiantz, A., *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 76*:5387-5391 (1979); Raff, M.C., *Science 243*:1450-1455 (1989)). Nevertheless, although extensive work has defined different glial subtypes and lineages (Raff, M.C., *Science 243*:1450-1455 (1989)), no systematic classification of regionally-specific glial subtypes has yet emerged. Based on our previous work, indicating that astrocyte subpopulations elaborate trophic factors in a regionally-specific fashion (Lu, B., *et al.*, *Exp. Neurol. 104*:191-199 (1989); O'Malley, E.K., *et al.*, *Brain Res. 582*:65-70 (1992)), we adopted a strategy of approaching glial typology according to the pattern of genes expressed. By combining the purification of region-specific glial subtypes in primary culture with current molecular strategies, we generated highly specific cDNA populations.

In sum, our initial studies suggest that type 1 astrocytes from different brain regions are heterogeneous, expressing different patterns of genes in a regionally-specific manner. Conversely, other genes are expressed virtually ubiquitously, suggesting commonality of particular type 1 functions in different brain areas.

The construction of cDNA libraries and the use of differential cloning techniques from trace amounts of RNA using the polymerase chain reaction have already been described (Belyavsky, A., *Nuc. Acids Res. 17*:2919-2932 (1989); Brunet, J.F., *et al.*, *Science 252*:856-859 (1991)). In contrast, we developed a non-sequence specific cDNA amplification method for use in subtractive hybridization. Consequently, we were able to access gene expression of highly purified cellular subpopulations in different brain regions. Our initial results provide new insights concerning astrocytic metabolic functions, and have identified novel genes differentially expressed by type 1 astrocytes in different regions.

The widespread elevation of AT1-40 (PMP70), which encodes a critical peroxisomal membrane protein, strongly suggests that this organelle is central to astrocytic 1 function throughout the brain. While the precise functions of peroxisomes remain to be elucidated, participation in specialized biosynthetic processes, such as bile-acid synthesis in liver, and catabolic reactions, including peroxide-based respiration, raise intriguing possibilities (see Valle and Gärtner (*Nature 361*:682-683 (1993))). Moreover, well-documented high expression of peroxisomes in liver and kidney suggests a role in detoxification and secretory functions. These considerations raise the possibility that astrocytic peroxisomes play a role in the blood-brain barrier, a contention we are presently investigating. Finally, our observations of high expression of PMP70 in type 1 astrocytes may be clinically relevant, since

mutations of the encoding gene are associated with cerebro-hepato-renal (Zellweger) syndrome (Gärtner, J., *et al.*, *Nature Genetics 1*:16-23 (1992)). It will now be of interest to assess astrocytic function in this debilitating disorder, and evaluate the role of astrocytic type 1 dysfunction in the pathogenesis of this disease. The observation that AT1-40 expression, though widespread, does vary regionally, suggests that regional metabolic requirements vary, and may explain regional susceptibility to dysfunction.

In addition to AT1-40, the PCR-based subtractive cloning procedure identified a number of novel astrocyte type 1 specific cDNA's. One example, AT1-46, may serve to indicate our findings and the directions of investigation. This cDNA recognizes a single copy gene which is expressed as a 4.8 kb low abundance transcript. A number of AT1-46 positive clones have been isolated from a cDNA library constructed from basal forebrain type 1 astrocytes and are currently being analyzed. Preliminary evidence derived from Northern analysis and *in situ* hybridization indicates that AT1-46 RNA is expressed in a regionally-specific, developmentally regulated fashion. In the P1 rat brain, the highest AT1-46 expression was detected in the olfactory neuroepithelium, caudate-putamen and septum as well as in the cingulate, frontal and parietal cortices. Additionally, lower levels of the AT1-46 transcript were identified in the hippocampus. The present approach, consequently, is identifying novel, as well as known genes, and regionally-specific, as well as widely expressed astrocyte gene products.

As determined using *in situ* analysis, the expression of AT1-46 is developmentally regulated in a region and cell specific fashion. The regions such as the various cortices and hippocampus express AT1-46 throughout postnatal development while in the septum expression is confined to P1. At P1 both glial and neuronal cells (hippocampus) appear to express AT1-46

while in the adult, expression is mainly limited to cells with neuronal morphology. AT1-46 is widely expressed in the adult but at different levels in various regions. The highest expression occurs in regions indicated above. There is lower expression in areas such as the caudate-putamen. Lastly, only a subpopulation of neurons express AT1-46 in the cortex or dentate gyrus. In regions such as the piriform cortex, cells with different morphologies appear to express AT1-46.

Although the PCR-based subtractive cloning proved useful to examine glial subtype gene expression it is not without limitations. The non-specific cDNA amplification requires the addition of homopolymeric tails to the cDNA termini. This creates the possibility that internal homopolymeric stretches, particularly G's or C's, may serve as primer binding sites resulting in preferential amplification. To avoid this bias, we utilized high primer annealing temperatures and restricted the number of PCR cycles. Moreover, by restricting cycle number, the PCR was restricted to the linear phase of exponential growth in which amplification is proportional to initial mRNA abundance (Chelly, J., *et al.*, *Nature 333*:858-860 (1988)). This approach yields results that faithfully reflect initial transcript abundance, while minimizing artifactual amplification.

In closing, we report the development of a sensitive cloning strategy employing trace amounts of starting material to generate subtracted cDNA libraries. Using this subtractive hybridization approach, we have initiated analysis of glial-subtype and regionally-specific gene expression in the brain.

Table 1: Partial Sequence Analysis of Nigral Astrocyte Type 1 Clones	
AT1-clone number*	Sequence Analysis/Identity**
c. 48 c. 21 c. 40 c. 4, 17, 20, 41, 42 c. 46 (SEQ ID NO:3) c. 911 (SEQ ID NO:4) c. 45 (SEQ ID NO:28 and 29)	18s rRNA 28s rRNA peroxisomal membrane protein (Kamijo *et al.*) contain a rat-specific repetitive insertion novel, type 1 astrocyte cDNA's
*AT1-cDNA's isolated by PCR-based subtractive cloning and positive for type 1 astrocyte differential expression, as determined by two rounds of colony hybridization, were chosen for sequence analysis. **Sequence of the AT1-cDNA ends was determined (100-200 bp) and analyzed as described in the Methods.	

Example 3

Isolation and characterization of cDNA sequences encoding important protein sequence motifs.

An analysis was preformed to identify conserved amino acid and nucleic acid sequence motifs which are associated with important structural, regulatory, or biological properties of evolutionarily conserved proteins. In order to accomplish this task, a class of important proteins, the TGF-betas, was chosen and amino acid sequences which were conserved amongst TGF-beta(1-3) and G-TSF were identified. These sequences were then compared to the amino acid sequence of the brain expressed neurotrophic factors, GDNF and ATF. The final amino acid sequence which was identified was then used to design amplification primers.

It was decided that the codons used in the primers for a particular amino acid would not specifically be the codons used in GDNF or ATF. This

method was an attempt to obtain nucleic acid sequences encoding important glial derived proteins which were not specifically members of the GDNF or ATF families of neurotrophic factors. Instead of choosing the nucleic acid sequence of ATF or GDNF for the identified protein motifs, a generalized mammalian codon preference for the amino acid sequence motifs was used in designing the amplification primers. It was reasoned that the importance lay in the protein sequence motif and not specifically in the nucleic acid sequence preference displayed in two members of glial derived neurotrophic factors. It is interesting to note that by following this procedures, several of the primers derived did not possess 100% homology with the nucleic acid sequence of GDNF or ATF.

The following amino acid sequences, and the corresponding amplification primers were used;

1) AGATCTAAGCTTYTNGTNYTNYTNCA (SEQ ID NO:16)

Based on amino acid sequence LVLLH (SEQ ID NO:20)

2) AAGATCCTCGAGNGCNACNGGNCGTRCARCA (SEQ ID NO:17)

Based on amino acid sequence CCRPVA (SEQ ID NO:21)

3) AGATCTAAGCTTGAYAARCARGCNGC (SEQ ID NO:18)

Based on amino acid sequence DKQAA (SEQ ID NO:22)

4) AAGATCCTCGAGDATRCANCCRCA (SEQ ID NO:19)

Based on amino acid sequence CGCI (SEQ ID NO:23)

The primers listed above contain sequences which facilitate cloning, for example restriction endonuclease cleavage site, as well as primer sequence corresponding to the identified target sequence.

In order to achieve optimum amplification the following conditions were chosen for the amplification parameters:

1.	3 cycles of	(a)	95°C; 1 minute
		(b)	Ramp to 37°C - 1 minute
		(c)	Annealing at 37°C - 1 minute
		(d)	Ramp to 72°C - 2 minutes 30 sec.
		(e)	72°C - 1 minute
2.	30 cycles of	(a)	95°C; 1 minute
		(b)	ramp to 51°C - 30 sec.
		(c)	Annealing at 51°C -- 1 minute
		(d)	Ramp to 72°C -- 1 minute
		(e)	72°C -- 1 minute
3.	Final step	10 minutes at 72°C.	

The resulting PCR products were either cloned directly with the "TA Cloning Kit" (invitrogen) or digested with XhoI and HindIII, restriction sites which were present in the PCR primers, and then ligated in the pBluescript plasmid.

Following this procedure, several important cDNA sequences have been obtained. These are presented in Table 2 and described below.

Cortical clones

These clones were isolated by cloning the PCR products generated from reverse transcribed cortical type 1 astrocyte RNA. The primers were the 5' and 3' TGF-β-like primers, which contain incorporated restriction sites for HindIII and XhoI, respectively. The restriction sites were used to clone the PCR products directly into the comparable sites of Bluescript pSK(-). These clones have been partially sequenced from the T7 polymerase primer site.

CtxG5.T7

```
1     gtggtgcagg gcctcaggga gctggaccct ctgcaagaca ccagcaaggc
51    tgtcactgaa gaaggcaagg taggaggcac agtaggcaga aaggaagctg
101   aatgtggcat agatgtatgt caggccagtg caaactgggc tgtaccacag
151   caagcaggta ggagccacag gcttgcaagg t     (SEQ ID NO:5)
```

CtxG7.T7

```
1     gatccacaag tttgccaaga agcagatatg ccctacgaaa gccaaagcaa
51    ggtgtacgaa tagacaagaa ttgaagagcc aacaactgtt gacgaggaac
101   tgatatataa gtgctaatga acgcttggat gcgctcttct ggagaaagca
151   gagcgaacac gttctttcc (SEQ ID NO:6)
```

CTX1-SP6

Amplified from cortical type 1 RNA with TGF beta-like primers TA cloned; Sp6 corresponds to the 3' TGF primer DS did minipreps/glycerol stocks

```
1     agcaacagga cggcagcaaa attgttggca atggagaact acaaagtgct
51    ttaaagcttc atccgctgat gaagctgtaa ctaagctcac acacacacaa
101   cttgccctga agtatttcta agtgtttgct aaattcaggg ggaaagtatt
151   tcgacactgg tgtcttggtc cacaagtaac attgggtata  aattcagtct
201   cttgcgtcca gcaggcactc gatgagtatg tgaacactaa tgagtga (SEQ ID
NO:7)
```

SN clones 1

These clones were isolated by cloning the PCR products generated from reverse transcribed substantia nigra type 1 astrocyte RNA . The primers were the 5' and 3' TGF-β-like primers, which contain incorporated restriction sites for HindIII and XhoI, respectively. The restriction sites were used to clone the PCR products directly into the comparable sites of Bluescript pSK(-).

The two clones below have been sequenced from both the T3 and T7 primers. In each case, the sequence at the end of each run overlaps. The information below represent a composite of each of the sequences in the T3 to T7 direction.

SND5.T3T7

```
1     ttcgatatca agcttttagt attatttcag actatagagc accctaccgt
51    tgtcaccttc atcagggtcc acggctttgg cctggaagat gctgtgtcct
101   gcctgccagt tctccactgc atctatgctt tccactgcct ggaggaagtg
151   gggggcgttg tcattcaaat ctttcactgt gatgttaacc acagcatctc
201   ctgtgactgc cccccnactg gccaccactc tcagctgata aaaggactgt
251   tcttccctgt caatga  (SEQ ID NO:7)
```

SND6.T3T7

```
1     aagcttctcg ttctattcca cgggccatgg cagtgaaacc tggcttcagt
51    tttgtttcca aaggacttct ttctccccca gatctgagat gtattcgaaa
101   ggcaagggtc ccacagaggc tccaaaatga aggcattagg aaaccggagt
151   gtcaacgggc tccctggtca gttcacctga gggatcgctg ctaaggggag
201   tagttcagag acatgagctt gggttttgtt agggtctgtt ttagggttat
251   tttattgggg tttggttctg ttgttgttgt tgttgttgtt gttgttgtag
301   cccagttgca ctcc  (SEQ ID NO:8)
```

SN clones 2

This clone was isolated by cloning the PCR products generated from reverse transcribed substantia nigra type 1 astrocyte RNA . The primers were the 5′ and 3′ TGF-β-like primers, which contain incorporated restriction sites for HindIII and XhoI, respectively. The restriction sites were used to clone the PCR product directly into the comparable sites of Bluescript pSK(-).

This clone has been sequenced from the T3 and T7 primer sites. However the sequences do not yet overlap. Since the database search suggests that this is probably the rat transcription factor TFE3 (due to its homology to murine TFE3), it appears that there are probably another 40, or so bases to go before the sequences overlap.

SNG8.T3 (rTFE3.T3?)

```
1     aagcttctcg tatttctaca aaagggcctt tgcctcggtt tccgagatct
51    cccgtttgat gttaggcagc tcagcaggac aggaattgct gacagtgatg
101   gctggggtgg ccacaccctg actgctgtac acatcaagta gatttcctga
151   cacaggcagc gtgctgggga gct  (SEQ ID NO:24)
```

SNG8.T7 (rTFE3.T7?)

```
1     cctcgagggc aaccgggcga caacaggtga aacagtactt gtctactaca
51    cttgggccca agctggcttc acaggccctc accccaccac cagggccttc
101   cagtgctcag ccacttcctg cccctgaaac tgctcatgcc accggcccta
151   caggcagtgc tcctaacagc cccatggcat tgctcaccat tgggtccagc
201   tcagagaagg agattgatga tgtcattgat gagatcatca gcctggagtc
(SEQ ID NO:25)
```

S1-T7

TGF beta like PCR TA clone, amp'd from SN type 1 RNA.T7 prime.

..

```
1     ttttggtact gctacagagt accaagaatg aaaaaaaagt ttcaactact
51    gagattttac tgagaagtca aaaccaaaaa atttttaga aagtgataga
101   aaaacaataa aatttctgta gctctctgat tatctatttt taaactggac
151   attagtgtgg tcacaggtga tcatttcaag gaggataatc agtgtgttca
201   aacatttgaa cagcctagac caatgaccca tgacat  (SEQ ID NO:9)
```

S1-SP6

TGF beta like degenerate PCR TA clone, amp'd from SN type 1 RNA

```
1     aagaycctcg agggccaccg gtctacaaca gatcatgaaa gtgttggatg
51    ctttggaaag ttaatgtaaa agaaagttat ttaagagaaa ttaaggcaac
101   gaagagaaac acgaaaatat atttcctgac tcaatactaa ccagagacct
151   ttgatttgct cacggggtgc ctgaggagag ggtctaaatg caaattattg
201   taatcaatct cgggatggta cacttttt  (SEQ ID NO:10)
```

Source Tissue	Sample #	Cloning Vector	Cloning Strategy	Length of Determined Sequence	Homology to database	ORFS	Identity
CTX	G5	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	181bp SEQ ID NO:5	none	yes 2	unknown
CTX	G6	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	193bp	yes - to numerous rRNAs	N.D.	rRNA
CTX	G7	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	169bp SEQ ID NO:6	none	yes 2	unknown
SN	D5	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	266bp SEQ ID NO:7	none	yes 1	unknown
SN	D6	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	314bp SEQ ID NO:8	none	N.D.	unknown

Source Tissue	Sample #	Cloning Vector	Cloning Strategy	Length of Determined Sequence	Homology to database	ORFS	Identity
SN	G8	psk- (bluescript)	incorporate HindIII and XhoI sites in primers	250bp→ SEQ ID NO:4 173bp← SEQ ID NO:25	Yes -- to mTFE3 and hTFE3	yes -one identical to mTFE3	rTFE3?
SN	S1	TA	TA	5'→236bp SEQ ID NO:9 3'→228bp SEQ ID NO:10	none	N.D.	unknown
CTX	C1	TA	TA	247bp SEQ ID NO:11	none	N.D.	unknown

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) INVENTORS: SCHAAR, DALE G.
DREYFUS, CHERYL F.
BLACK, IRA B.

(iii) TITLE OF INVENTION: NOVEL NUCLEIC ACID SEQUENCES ISOLATED FROM GLIAL CELLS

(iv) NUMBER OF SEQUENCES: 41

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(vi) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vii) CURRENT INTERNATIONAL APPLICATION DATA:
(A) APPLICATION NUMBER: (To Be Assigned)
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(viii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: Not Yet Assigned
(B) FILING DATE: 20-Dec-1994
(C) CLASSIFICATION:

(ix) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER: 08/275,709
(B) FILING DATE: 18-July-1994
(C) CLASSIFICATION:

(x) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/172,327
(B) FILING DATE: 22-Dec-1993
(C) CLASSIFICATION:

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(C) REFERENCE/DOCKET NUMBER: 1459.004002

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 622 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 50..604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GGTCTACGGA GACCGGATCC GAGGTGCCGC CGCCGGACGG GACTCTAAG ATG AAG
       55                                                  Met Lys
                                                             1

TTA TGG GAT GTC GTG GCT GTC TGC CTG GTG TTG CTC CAC ACC GCG TCT
     103
Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr Ala Ser

          5                  10                  15

GCC TTC CCG CTG CCC GCC GCC AAT ATG CCC GAA GAT TAT CCT GAC CAG
     151
Ala Phe Pro Leu Pro Ala Ala Asn Met Pro Glu Asp Tyr Pro Asp Gln

     20                  25                  30

TTT GAT GAC GTC ATG GAT TTT ATT CAA GCC ACC ATC AAA AGA CTG AAA
     199
Phe Asp Asp Val Met Asp Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys

 35                  40                  45                  50

AGG TCA CCA GAT AAA CAA GCG GCG GCA CTT CCT CGA AGA GAG AGG AAC
     247
Arg Ser Pro Asp Lys Gln Ala Ala Ala Leu Pro Arg Arg Glu Arg Asn

                 55                  60                  65

CGG CAA GCT GCA GCT GCC AGC CCA GAG AAT TCC AGA GGG AAA GGT CGC
     295
Arg Gln Ala Ala Ala Ala Ser Pro Glu Asn Ser Arg Gly Lys Gly Arg

             70                  75                  80
```

```
AGA GGC CAG AGG GGC AAA AAT CGG GGG TGC GTC TTA ACT GCA ATA CAC
     343
Arg Gly Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His

         85                  90                  95

TTA AAT GTC ACT GAC TTG GGT TTG GGC TAC GAA ACC AAG GAG GAA CTG
     391
Leu Asn Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu

    100                 105                 110

ATC TTT CGA TAT TGT AGC GGT TCC TGT GAA GCG GCC GAG ACA ATG TAC
     439
Ile Phe Arg Tyr Cys Ser Gly Ser Cys Glu Ala Ala Glu Thr Met Tyr

115                 120                 125                 130

GAC AAA ATA CTA AAA AAT CTG TCT CGA AGT AGA AGG CTA ACA AGT GAC
     487
Asp Lys Ile Leu Lys Asn Leu Ser Arg Ser Arg Arg Leu Thr Ser Asp

                135                 140                 145

AAG GTA GGC CAG GCA TGT TGC AGG CCG GTC GCC TTC GAC GAC GAC CTG
     535
Lys Val Gly Gln Ala Cys Cys Arg Pro Val Ala Phe Asp Asp Asp Leu

            150                 155                 160

TCG TTT TTA GAC GAC AGC CTG GTT TAC CAT ATC CTA AGA AAG CAT TCC
     583
Ser Phe Leu Asp Asp Ser Leu Val Tyr His Ile Leu Arg Lys His Ser

        165                 170                 175

GCT AAA CGG TGT GGA TGT ATC TGACCCTGGC TCCAGAGA
      622
Ala Lys Arg Cys Gly Cys Ile
    180                 185
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr
 1               5                  10                  15

Ala Ser Ala Phe Pro Leu Pro Ala Ala Asn Met Pro Glu Asp Tyr Pro
            20                  25                  30

Asp Gln Phe Asp Asp Val Met Asp Phe Ile Gln Ala Thr Ile Lys Arg
        35                  40                  45

Leu Lys Arg Ser Pro Asp Lys Gln Ala Ala Ala Leu Pro Arg Arg Glu
    50                  55                  60

Arg Asn Arg Gln Ala Ala Ala Ala Ser Pro Glu Asn Ser Arg Gly Lys
65                  70                  75                  80

Gly Arg Arg Gly Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala
                85                  90                  95

Ile His Leu Asn Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu
            100                 105                 110

Glu Leu Ile Phe Arg Tyr Cys Ser Gly Ser Cys Glu Ala Ala Glu Thr
        115                 120                 125

Met Tyr Asp Lys Ile Leu Lys Asn Leu Ser Arg Ser Arg Arg Leu Thr
    130                 135                 140

Ser Asp Lys Val Gly Gln Ala Cys Cys Arg Pro Val Ala Phe Asp Asp
145                 150                 155                 160

Asp Leu Ser Phe Leu Asp Asp Ser Leu Val Tyr His Ile Leu Arg Lys
                165                 170                 175

His Ser Ala Lys Arg Cys Gly Cys Ile
            180                 185
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 831 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AAGCAGTTCT CCCAGCAGGA AGAGAAGAGG CAGAAGGCGG AGAGGCTGCA GCAGCAGCAG
    60
```

AAACACGAGA ACCAGATGCG AGACATGGTG GCACAGTGCG AGATCAAACC GAACGAGCTG
120

CAGCAGCTGC AGAATGAAAA GTGTCATCTG TTAGTGGAGC ATGAAACCCA GAAGCTGAAG
180

GCCCTGGACG AGAGCCATAA CCAGAGCCTG AGGGAATGGC GAGACAAGCT TCGGCCACGC
240

AAAAAGGGCC TGGAAGAGGA TTTGAACCAG AAGAAGCGGG AACAGGAAAT GTTCTTCAGA
300

CTAAGTGAGG AGGCAGAGAC CAGACCCACC ACACCCAACA GAGCCAGCAA GTTCTCCCCC
360

TACAGCTCTG GGGATGCTTC CTAACACACA CATGCCTGGG CTGCGGTGCG GCTGTACAGC
420

CACCAGGGCC ACCAACCCTC TACGAACAAG TGACTCAGGA CCTCTTCTCT TGCTTCTGTG
480

CCAGCTCCAA CTACCAGCAC CCAGTGCCAC AGCGCACCAG TGCTCTGATG ATGACTCATC
540

CAACTCAGAT CCATCACTAA AGTGACTGCT GTGGTCGGAC TGAGCGGATG GCGTACCTCC
600

TATTCGCCAA ACACCAGCTC TACTGTCTGT GGCACAAGCG CTACTGATGA CATCACACGA
660

ACCCATCCTT ATTGTGATCC TTGTGGTTTT TTCTTCTCCT TCAGTAATTC CTCACAGTGT
720

TGGAAAACAT CCCTCAGAGC CATTTTGCTT CTCAGCAGCC AGCTCTCAGG GGTGTCCCCA
780

TTACCCTCGT TCGCACAGCT GACTTTGTGC TCGATGAGAC GCTGTGTATG A
831

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2269 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCACGAGAA AAGGCCCTGG AAGAGGATTT GAACCAGAAG AAGCGGGAAC AGGAAATGTT
60

CTTCAGACTA AGTGAGGAGG CAGAGACCAG ACCCACCACA CCCAACAGAG CCAGCAAGTT
120

CTTCCCCTAC AGCTCTGGGG ATGCTTCCTA ACACACACAT GCCTGGGCTG CGGTGCGGCA
180

GTACAGCCAC CAGGGCCACC AACCCTCTAC AAACAAGTGA CTCAGGACCT CTTCCTCTTG
240

CTTCTGTGCC AGCTCCAACT ACCCAGCACC CCAGTTGCCC ACAGCACCAC CCCAGTGCTT
300

CTGATGGATG ACCTCATCCC AACTCAGATT CCCATCACCT GGAAGTGACC TGGGCTGTTG
360

GGGTCCGGGA CCGAGCGGGA TGGGCGTACC CCTCCTGTTT GCCAAAACAC CAGCTCTACT
420

GTCTGTGGGC ACAAGCGCTA CTGATGACAT CACCACGAAC CCATCCTTAT TGTGATCCTT
480

GTGGTTTTTT CTTCTCCTTC AGTAATTCCT CACAGTGTTG GAAAACATCC CTCAGAGCCA
540

TTTTGCTTCT CAGCAGCCAG CTCTCAGGGG TGTCCCCATT ACCCTGCTTC GCACAGCTGA
600

CTTTGTGCTC GATGAGACGC TGTGTATGTG GGGGTAGGGA GTGGGGAAAG GGAGGCCAGA
660

AATGTTCATT CTGCTGGTTT CTGACATTTT ATGCCATCTC ATTTTGCCTC TCCCTGTCAC
720

ACACACACAC ACACACACAC ACACACACAC ACACACACAC ACACACACAA TGCAAACACA
780

CAACTTGGCC CTCCTGAACC TGATCGTAGG ACACGGAGTA CAGAGCATGT CAGGTGGAGC
840

AGCTGCTGGG GCATGCTGAG TGCTGGCCCC AAAGCCCAGA GAAGGCACAG GCTGTACTGC
900

AGCCTGCCTG CCACTCGTTT GGCTGCACAC AGGATCCTGT GTTCAGGGGT AAACTCCCCT
960

CCACACTTGT CTTCTGCTGC CTAGCGCATG CCAATCTCGC CCTTGCCCAG TTGTTGGCAA
1020

GTACTGGGGA AGGCTCCTGA CCTTTGACCT TTGCCCCAGT CCTGCACTGG AGTCCCACTG
1080

TACATTTCCA CTAAGCCGGA CAGTCCTTTG GACTTCTCTG TTTAGGAAGA GATGCTTCCC
1140

ACCCCTGGGA ACAGCCGAAG CTCAGGAAAA TGCCAAGCCT CGTGCCTGGG CCTTTGGGTT
1200

GCTCAGGTAG CCTCCCAAGA TGCTGCGCCC CATAGGCTAC CATGCCCAGA AAAGCAGCTG
1260

GTCGGCCCAG CCGGCGTTCG CTGATAGCGC CTTAGGGCTC AGTTAAAGCA CAGGTAAATG
1320

GCTGGCTGCT TTGTATACCC TCCTTTTAGA CAGCATCACC CCAGGGATTA GGATGGGATG
1380

GGTGGGGGCG GGGCACCCAG GCAGTGGAGT CTGGGAGTGG CTGAGACCTC AGCAGTATTT
1440

CCCCATCACT GCCCCATGCT GAGACAACCT TCTAGGACGT TTCCTCAGAT GCTGACTGGG
1500

TGCTTGGGAG GGGAGTGGGC TAGTAAAACA AAATAGGAAA ACAGGTCTTG GGACTCCCAG
1560

ATCTTGTGTG CAGTAAGGAA GTTCACAGAG CCCCAGGAAG GCGATAGTTC TCAGGGTAGC
1620

GAGCGTCAGC TTGCTTTCAG GCCGCACACC GAGGAGTCTT GAGGAACAGT TGACTTCTTT
1680

CTTACTGGTG CATGGGGGCT GGGAAACACA AGTTGTCAGA GTGCAGCTGT GGGACTCAGA
1740

GATGGGAAGT GGGCAAGGCC ACGCCCTGCA GGGCTCTACC ATTGTTTACA ATGTACTTGG
1800

CTGCATTCGG GGGTGGGGGG AACTTGACAG TGGCTATTAG GCAAAATGCC GGTTTTGTGG
1860

TTCAGGTAAC AGTCTTTGAC CACTCCCTGA CGTCATTCGT ACTGTCCTCC TCCTTGTTGC
1920

TTCCACACTT AGTCCCACCT GAGCTCTGGT ACCTCTGCTG TGCCTTTTTT GAGTGGGGTC
1980

TAGCCTTGTC TTCCAGCCTC ATAATTTAAC CTAAGTGCAA TGCCTGCCAC CGACAAAGGC
2040

CCGTGAAGTA TTCCTCATGT CCTGTGCTAA CGTTTTCTGT ATAGGAACAG GCAGAAATGT
2100

CTTTAGCACC GCGGATATAA CTAACTTATA TTTCCCTTCA CGAAGGATAG AAGTAACGGG
2160

TGTGTCATTT CCAACGGTCA TGTATAATTT TTGTAAACTG TTCTCTGCAA ACAAAAAAAA
2220

TGTAAATATG CTTCTAATAA AATAATAAGG TAAAAAAAAA AAAAAAAAA
2269

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGGTGCAGG GCCTCAGGGA GCTGGACCCT CTGCAAGACA CCAGCAAGGC TGTCACTGAA
60

GAAGGCAAGG TAGGAGGCAC AGTAGGCAGA AAGGAAGCTG AATGTGGCAT AGATGTATGT
120

CAGGCCAGTG CAAACTGGGC TGTACCACAG CAAGCAGGTA GGAGCCACAG GCTTGCAAGG
180

T
181

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCCACAAG TTTGCCAAGA AGCAGATATG CCCTACGAAA GCCAAAGCAA GGTGTACGAA
60

TAGACAAGAA TTGAAGAGCC AACAACTGTT GACGAGGAAC TGATATATAA GTGCTAATGA
120

ACGCTTGGAT GCGCTCTTCT GGAGAAAGCA GAGCGAACAC GTTCTTTCC
169

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 265 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTCGATATCA AGCTTTTAGT ATTATTTCAG ACTATAGAGC ACCCTACCGT TGTCACCTTC
60

ATCAGGGTCC ACGGCTTTGG CCTGGAAGAT GCTGTGTCCT GCCTGCCAGT TCTCCACTGC
120

ATCTATGCTT TCCACTGCCT GGAGGAAGTG GGGGGCGTTG TCATTCAAAT CTTTCACTGT
180

GATGTTAACC ACAGCATCTC CTGTGACTGC CCCCCACTGG CCACCACTCT CAGCTGATAA
240

AAGGACTGTT CTTCCCTGTC AATGA
265

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 314 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGCTTCTCG TTCTATTCCA CGGGCCATGG CAGTGAAACC TGGCTTCAGT TTTGTTTCCA
60

AAGGACTTCT TTCTCCCCCA GATCTGAGAT GTATTCGAAA GGCAAGGGTC CCACAGAGGC
120

TCCAAAATGA AGGCATTAGG AAACCGGAGT GTCAACGGGC TCCCTGGTCA GTTCACCTGA
180

GGGATCGCTG CTAAGGGGAG TAGTTCAGAG ACATGAGCTT GGGTTTTGTT AGGGTCTGTT
240

TTAGGGTTAT TTTATTGGGG TTTGGTTCTG TTGTTGTTGT TGTTGTTGTT GTTGTTGTAG
300

CCCAGTTGCA CTCC
314

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 236 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTGGTACT GCTACAGAGT ACCAAGAATG AAAAAAAAGT TTCAACTACT GAGATTTTAC
60

TGAGAAGTCA AAACCAAAAA ATTTTTTAGA AAGTGATAGA AAAACAATAA AATTTCTGTA
120

GCTCTCTGAT TATCTATTTT TAAACTGGAC ATTAGTGTGG TCACAGGTGA TCATTTCAAG
180

GAGGATAATC AGTGTGTTCA AACATTTGAA CAGCCTAGAC CAATGACCCA TGACAT
236

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 227 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGACCTCGA GGGCCACCGG TCTACAACAG ATCATGAAAG TGTTGGATGC TTTGGAAAGT
60

TAATGTAAAA GAAAGTTATT TAAGAGAAAT TAAGGCAACG AAGAGAAACA CGAAAATATA
120

TTTCCTGACT CAATACTAAC CAGAGACCTT TGATTTGCTC ACGGGGTGCC TGAGGAGAGG
180

GTCTAAATGC AAATTATTGT AATCAATCTC GGGATGGTAC ACTTTTT
227

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 247 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCAACAGGA CGGCAGCAAA ATTGTTGGCA ATGGAGAACT ACAAAGTGCT TTAAAGCTTC
60

ATCCGCTGAT GAAGCTGTAA CTAAGCTCAC ACACACACAA CTTGCCCTGA AGTATTTCTA
120

AGTGTTTGCT AAATTCAGGG GGAAAGTATT TCGACACTGG TGTCTTGGTC CACAAGTAAC
180

ATTGGGTATA AATTCAGTCT CTTGCGTCCA GCAGGCACTC GATGAGTATG TGAACACTAA
240

TGAGTGA
247

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTCTACGGA GACCGGATCC GAGGTGC
27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCTCTGGAGC CAGGGTCAGA TACATC
26

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGGCTCGAG CGGCCGCT
18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGATCTAAGC TTYTNGTNYT NYTNCA
26

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGATCTAAGC TTYTNGTNYT NYTNCA
26

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAGATCCTCG AGNGCNACNG GNCGTRCARC A
31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGATCTAAGC TTGAYAARCA RGCNGC
26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGATCCTCG AGDATRCANC CRCA
24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Val Leu Leu His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Cys Cys Arg Pro Val Ala
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asp Lys Gln Ala Ala
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Cys Gly Cys Ile
1

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 173 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAGCTTCTCG TATTTCTACA AAAGGGCCTT TGCCTCGGTT TCCGAGATCT CCCGTTTGAT
60

GTTAGGCAGC TCAGCAGGAC AGGAATTGCT GACAGTGATG GCTGGGGTGG CCACACCCTG
120

ACTGCTGTAC ACATCAAGTA GATTTCCTGA CACAGGCAGC GTGCTGGGGA GCT
173

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 250 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCTCGAGGGC AACCGGGCGA CAACAGGTGA AACAGTACTT GTCTACTACA CTTGGGCCCA
60

AGCTGGCTTC ACAGGCCCTC ACCCCACCAC CAGGGCCTTC CAGTGCTCAG CCACTTCCTG
120

CCCCTGAAAC TGCTCATGCC ACCGGCCCTA CAGGCAGTGC TCCTAACAGC CCCATGGCAT
180

TGCTCACCAT TGGGTCCAGC TCAGAGAAGG AGATTGATGA TGTCATTGAT GAGATCATCA
240

GCCTGGAGTC
250

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 700 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGTCTACGGA GACCGGATCC GAGGTGCCGC CGCCGGACGG GACTCTAAGA TGAAGTTATG
60

GGATGTCGTG GCTGTCTGCC TGGTGTTGCT CCACACCGCG TCTGCCTTCC CGCTGCCCGC
120

CGGTAAGAGG CTTCTCGAAG CGCCCGCCGA AGACCACTCC CTCGGCCACC GCCGCGTGCC
180

CTTCGCGCTG ACCAGTGACT CCAATATGCC CGAAGATTAT CCTGACCAGT TTGATGACGT
240

CATGGATTTT ATTCAAGCCA CCATCAAAAG ACTGAAAAGG TCACCAGATA AACAAGCGGC
300

GGCACTTCCT CGAAGAGAGA GGAACCGGCA AGCTGCAGCT GCCAGCCCAG AGAATTCCAG
360

AGGGAAAGGT CGCAGAGGCC AGAGGGGCAA AAATCGGGGG TGCGTCTTAA CTGCAATACA
420

CTTAAATGTC ACTGACTTGG GTTTGGGCTA CGAAACCAAG GAGGAACTGA TCTTTCGATA
480

TTGTAGCGGT TCCTGTGAAG CGGCCGAGAC AATGTACGAC AAAATACTAA AAAATCTGTC
540

TCGAAGTAGA AGGCTAACAA GTGACAAGGT AGGCCAGGCA TGTTGCAGGC CGGTCGCCTT
600

CGACGACGAC CTGTCGTTTT TAGACGACAG CCTGGTTTAC CATATCCTAA GAAAGCATTC
660

CGCTAAACGG TGTGGATGTA TCTGACCCTG GCTCCAGAGA
700

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr
1 5 10 15

Ala Ser Ala Phe Pro Leu Pro Ala Gly Lys Arg Leu Leu Glu Ala Pro
20 25 30

```
Ala Glu Asp His Ser Leu Gly His Arg Arg Val Pro Phe Ala Leu Thr
        35                  40                  45

Ser Asp Ser Asn Met Pro Glu Asp Tyr Pro Asp Gln Phe Asp Asp Val
    50                  55                  60

Met Asp Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys Arg Ser Pro Asp
65                  70                  75                  80

Lys Gln Ala Ala Ala Leu Pro Arg Arg Glu Arg Asn Arg Gln Ala Ala
                85                  90                  95

Ala Ala Ser Pro Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly Gln Arg
            100                 105                 110

Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His Leu Asn Val Thr
        115                 120                 125

Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe Arg Tyr
    130                 135                 140

Cys Ser Gly Ser Cys Glu Ala Ala Glu Thr Met Tyr Asp Lys Ile Leu
145                 150                 155                 160

Lys Asn Leu Ser Arg Ser Arg Arg Leu Thr Ser Asp Lys Val Gly Gln
                165                 170                 175

Ala Cys Cys Arg Pro Val Ala Phe Asp Asp Asp Leu Ser Phe Leu Asp
            180                 185                 190

Asp Ser Leu Val Tyr His Ile Leu Arg Lys His Ser Ala Lys Arg Cys
        195                 200                 205

Gly Cys Ile
    210
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AAAAAAAAAG AAAAAAAGAA TTCTAGCAAA TAAAACGTCA GCTTCCTGAA AGTACTGTCC
60

ACCCTGACCA AATAAGACCA GGAGCACAGG CCTTTGGGGA TTTAGCCTCA GTGGTAGAG
119

(2) INFORMATION FOR SEQ ID NO:29:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTTTTTTTT TTTTTTTTTT TTTTTTTTAG TCGGTGGTGG TGGTGGTGGG GAGGAGTACG
60

AAATACCGCA TGACAGACCT GAACGGAATC CGAGTTCGGA AGAACT
106

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 622 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 50..604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTCTACGGA GACCGGATCC GAGGTGCCGC CGCCGGACGG GACTTTAAG ATG AAG
55
Met Lys
1

TTA TGG GAT GTC GTG GCT GTC TGC CTG GTG CTG CTC CAC ACC GCG TCC
103
Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr Ala Ser
5 10 15

GCC TTC CCG CTG CCC GCC GCA AAT ATG CCA GAG GAT TAT CCT GAT CAG
151

```
Ala Phe Pro Leu Pro Ala Ala Asn Met Pro Glu Asp Tyr Pro Asp Gln
     20                  25                  30

TTC GAT GAT GTC ATG GAT TTT ATT CAA GCC ACC ATT AAA AGA CTG AAA
     199
Phe Asp Asp Val Met Asp Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys
 35                  40                  45                  50

AGG TCA CCA GAT AAA CAA ATG GCA GTG CTT CCT AGA AGA GAG CGG AAT
     247
Arg Ser Pro Asp Lys Gln Met Ala Val Leu Pro Arg Arg Glu Arg Asn
                 55                  60                  65

CGG CAG GCT GCA GCT GCC AAC CCA GAG AAT TCC AGA GGA AAA GGT CGG
     295
Arg Gln Ala Ala Ala Ala Asn Pro Glu Asn Ser Arg Gly Lys Gly Arg
             70                  75                  80

AGA GGC CAG AGG GGC AAA AAC CGG GGT TGT GTC TTA ACT GCA ATA CAT
     343
Arg Gly Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His
         85                  90                  95

TTA AAT GTC ACT GAC TTG GGT CTG GGC TAT GAA ACC AAG GAG GAA CTG
     391
Leu Asn Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu
    100                 105                 110


ATT TTT AGG TAC TGC AGC GGC TCT TGC GAT GCA GCT GAG ACA ACG TAC
     439
Ile Phe Arg Tyr Cys Ser Gly Ser Cys Asp Ala Ala Glu Thr Thr Tyr
115                 120                 125                 130

GAC AAA ATA TTG AAA AAC TTA TCC AGA AAT AGA AGG CTG GTG AGT GAC
     487
Asp Lys Ile Leu Lys Asn Leu Ser Arg Asn Arg Arg Leu Val Ser Asp
                135                 140                 145
```

```
AAA GTA GGG CAG GCA TGT TGC AGA CCC ATC GCC TTT GAT GAT GAC CTG
     535
Lys Val Gly Gln Ala Cys Cys Arg Pro Ile Ala Phe Asp Asp Asp Leu

            150                 155                 160


TCG TTT TTA GAT GAT AAC CTG GTT TAC CAT ATT CTA AGA AAG CAT TCC
     583
Ser Phe Leu Asp Asp Asn Leu Val Tyr His Ile Leu Arg Lys His Ser

        165                 170                 175


GCT AAA AGG TGT GGA TGT ATC TGACCCTGGC TCCAGAGA
      622
Ala Lys Arg Cys Gly Cys Ile
    180                 185
```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```
Met Lys Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr
  1               5                  10                  15

Ala Ser Ala Phe Pro Leu Pro Ala Ala Asn Met Pro Glu Asp Tyr Pro
            20                  25                  30

Asp Gln Phe Asp Asp Val Met Asp Phe Ile Gln Ala Thr Ile Lys Arg
        35                  40                  45

Leu Lys Arg Ser Pro Asp Lys Gln Met Ala Val Leu Pro Arg Arg Glu
    50                  55                  60

Arg Asn Arg Gln Ala Ala Ala Ala Asn Pro Glu Asn Ser Arg Gly Lys
65                  70                  75                  80

Gly Arg Arg Gly Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala
                85                  90                  95

Ile His Leu Asn Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu
            100                 105                 110
```

Glu Leu Ile Phe Arg Tyr Cys Ser Gly Ser Cys Asp Ala Ala Glu Thr
115 120 125

Thr Tyr Asp Lys Ile Leu Lys Asn Leu Ser Arg Asn Arg Arg Leu Val
130 135 140

Ser Asp Lys Val Gly Gln Ala Cys Cys Arg Pro Ile Ala Phe Asp Asp
145 150 155 160

Asp Leu Ser Phe Leu Asp Asp Asn Leu Val Tyr His Ile Leu Arg Lys
165 170 175

His Ser Ala Lys Arg Cys Gly Cys Ile
180 185

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 474 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 58..456

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGTCTACGGA GACCGGATCC GAGGTGCCGC CGCCGGACGG GACTTTAAGA TGAAGTT
57

ATG GGA TGT CGT GGC TGT CTG CCT GGT GCT GCT CCA CAC CGC GTC CGC
105
Met Gly Cys Arg Gly Cys Leu Pro Gly Ala Ala Pro His Arg Val Arg
1 5 10 15

CTT CCC GCT GCC AAC CCA GAG AAT TCC AGA GGA AAA GGT CGG AGA GGT
153
Leu Pro Ala Ala Asn Pro Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly
20 25 30

CAG AGG GGC AAA AAC CGG GGT TGT GTC TTA ACT GCA ATA CAT TTA AAT
201

```
Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His Leu Asn
         35                  40                  45

GTC ACT GAC TTG GGT CTG GGC TAT GAA ACC AAG GAG GAA CTG ATT TTT
     249
Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe
     50                  55                  60

AGG TAC TGC AGC GGC TCT TGC GAT GCA GCT GAG ACA ACG TAC GAC AAA
     297
Arg Tyr Cys Ser Gly Ser Cys Asp Ala Ala Glu Thr Thr Tyr Asp Lys
 65                  70                  75                  80

ATA TTG AAA AAC TTA TCC AGA AAT AGA AGG CTG GTG AGT GAC AAA GTA
     345
Ile Leu Lys Asn Leu Ser Arg Asn Arg Arg Leu Val Ser Asp Lys Val
                 85                  90                  95

GGG CAG GCA TGT TGC AGA CCC ATC GCC TTT GAT GAT GAC CTG TCG TTT
     393
Gly Gln Ala Cys Cys Arg Pro Ile Ala Phe Asp Asp Asp Leu Ser Phe
            100                 105                 110

TTA GAT GAT AAC CTG GTT TAC CAT ATT CTA AGA AAG CAT TCC GCT AAA
     441
Leu Asp Asp Asn Leu Val Tyr His Ile Leu Arg Lys His Ser Ala Lys
        115                 120                 125

AGG TGT GGA TGT ATC TGACCCTGGC TCCAGAGA
      474
Arg Cys Gly Cys Ile
    130
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Gly Cys Arg Gly Cys Leu Pro Gly Ala Ala Pro His Arg Val Arg
 1               5                  10                  15

Leu Pro Ala Ala Asn Pro Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly
            20                  25                  30

Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His Leu Asn
        35                  40                  45

Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe
    50                  55                  60

Arg Tyr Cys Ser Gly Ser Cys Asp Ala Ala Glu Thr Thr Tyr Asp Lys
65                  70                  75                  80

Ile Leu Lys Asn Leu Ser Arg Asn Arg Arg Leu Val Ser Asp Lys Val
                85                  90                  95

Gly Gln Ala Cys Cys Arg Pro Ile Ala Phe Asp Asp Asp Leu Ser Phe
            100                 105                 110

Leu Asp Asp Asn Leu Val Tyr His Ile Leu Arg Lys His Ser Ala Lys
        115                 120                 125

Arg Cys Gly Cys Ile
    130
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 700 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 50..682

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
GGTCTACGGA GACCGGATCC GAGGTGCCGC CGCCGGACGG GACTTTAAG ATG AAG
       55
                                                      Met Lys
                                                        1
```

```
TTA TGG GAT GTC GTG GCT GTC TGC CTG GTG CTG CTC CAC ACC GCG TCC
     103
Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr Ala Ser
          5                  10                  15

GCC TTC CCG CTG CCC GCC GGT AAG AGG CCT CCC GAG GCG CCC GCC GAA
     151
Ala Phe Pro Leu Pro Ala Gly Lys Arg Pro Pro Glu Ala Pro Ala Glu
     20                  25                  30

GAC CGC TCC CTC GGC CGC CGC CGC GCG CCC TTC GCG CTG AGC AGT GAC
     199
Asp Arg Ser Leu Gly Arg Arg Arg Ala Pro Phe Ala Leu Ser Ser Asp
 35                  40                  45                  50

TCA AAT ATG CCA GAG GAT TAT CCT GAT CAG TTC GAT GAT GTC ATG GAT
     247
Ser Asn Met Pro Glu Asp Tyr Pro Asp Gln Phe Asp Asp Val Met Asp
                 55                  60                  65

TTT ATT CAA GCC ACC ATT AAA AGA CTG AAA AGG TCA CCA GAT AAA CAA
     295
Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys Arg Ser Pro Asp Lys Gln
             70                  75                  80

ATG GCA GTG CTT CCT AGA AGA GAG CGG AAT CGG CAG GCT GCA GCT GCC
     343
Met Ala Val Leu Pro Arg Arg Glu Arg Asn Arg Gln Ala Ala Ala Ala
          85                  90                  95

AAC CCA GAG AAT TCC AGA GGA AAA GGT CGG AGA GGC CAG AGG GGC AAA
     391
Asn Pro Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly Gln Arg Gly Lys
     100                 105                 110

AAC CGG GGT TGT GTC TTA ACT GCA ATA CAT TTA AAT GTC ACT GAC TTG
     439
Asn Arg Gly Cys Val Leu Thr Ala Ile His Leu Asn Val Thr Asp Leu
115                 120                 125                 130
```

```
GGT CTG GGC TAT GAA ACC AAG GAG GAA CTG ATT TTT AGG TAC TGC AGC
     487
Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe Arg Tyr Cys Ser
                135                 140                 145

GGC TCT TGC GAT GCA GCT GAG ACA ACG TAC GAC AAA ATA TTG AAA AAC
     535
Gly Ser Cys Asp Ala Ala Glu Thr Thr Tyr Asp Lys Ile Leu Lys Asn
            150                 155                 160

TTA TCC AGA AAT AGA AGG CTG GTG AGT GAC AAA GTA GGG CAG GCA TGT
     583
Leu Ser Arg Asn Arg Arg Leu Val Ser Asp Lys Val Gly Gln Ala Cys
        165                 170                 175

TGC AGA CCC ATC GCC TTT GAT GAT GAC CTG TCG TTT TTA GAT GAT AAC
     631
Cys Arg Pro Ile Ala Phe Asp Asp Asp Leu Ser Phe Leu Asp Asp Asn
    180                 185                 190

CTG GTT TAC CAT ATT CTA AGA AAG CAT TCC GCT AAA AGG TGT GGA TGT
     679
Leu Val Tyr His Ile Leu Arg Lys His Ser Ala Lys Arg Cys Gly Cys
195                 200                 205                 210

ATC TGA CCC TGC CTC CAG AGA
      700
Ile
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Met Lys Leu Trp Asp Val Val Ala Val Cys Leu Val Leu Leu His Thr
  1               5                  10                  15
```

Ala Ser Ala Phe Pro Leu Pro Ala Gly Lys Arg Pro Pro Glu Ala Pro
20 25 30

Ala Glu Asp Arg Ser Leu Gly Arg Arg Arg Ala Pro Phe Ala Leu Ser
35 40 45

Ser Asp Ser Asn Met Pro Glu Asp Tyr Pro Asp Gln Phe Asp Asp Val
50 55 60

Met Asp Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys Arg Ser Pro Asp
65 70 75 80

Lys Gln Met Ala Val Leu Pro Arg Arg Glu Arg Asn Arg Gln Ala Ala
85 90 95

Ala Ala Asn Pro Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly Gln Arg
100 105 110

Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His Leu Asn Val Thr
115 120 125

Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe Arg Tyr
130 135 140

Cys Ser Gly Ser Cys Asp Ala Ala Glu Thr Thr Tyr Asp Lys Ile Leu
145 150 155 160

Lys Asn Leu Ser Arg Asn Arg Arg Leu Val Ser Asp Lys Val Gly Gln
165 170 175

Ala Cys Cys Arg Pro Ile Ala Phe Asp Asp Asp Leu Ser Phe Leu Asp
180 185 190

Asp Asn Leu Val Tyr His Ile Leu Arg Lys His Ser Ala Lys Arg Cys
195 200 205

Gly Cys Ile
210

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 522 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 28..504

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
GTCTACGGAG ACCGGATCCG AGCCAAT ATG CCC GAA GAT TAT CCT GAC CAG
      51
                              Met Pro Glu Asp Tyr Pro Asp Gln
                                1               5

TTT GAT GAC GTC ATG GAT TTT ATT CAA GCC ACC ATC AAA AGA CTG AAA
      99
Phe Asp Asp Val Met Asp Phe Ile Gln Ala Thr Ile Lys Arg Leu Lys
     10                  15                  20

AGG TCA CCA GAT AAA CAA GCG GCG GCA CTT CCT CGA AGA GAG AGG AAC
     147
Arg Ser Pro Asp Lys Gln Ala Ala Ala Leu Pro Arg Arg Glu Arg Asn
 25                  30                  35                  40

CGG CAA GCT GCA GCT GCC AGC CCA GAG AAT TCC AGA GGG AAA GGT CGC
     195
Arg Gln Ala Ala Ala Ala Ser Pro Glu Asn Ser Arg Gly Lys Gly Arg
                 45                  50                  55

AGA GGC CAG AGG GGC AAA AAT CGG GGG TGC GTC TTA ACT GCA ATA CAC
     243
Arg Gly Gln Arg Gly Lys Asn Arg Gly Cys Val Leu Thr Ala Ile His
             60                  65                  70

TTA AAT GTC ACT GAC TTG GGT TTG GGC TAC GAA ACC AAG GAG GAA CTG
     291
Leu Asn Val Thr Asp Leu Gly Leu Gly Tyr Glu Thr Lys Glu Glu Leu
         75                  80                  85

ATC TTT CGA TAT TGT AGC GGT TCC TGT GAA GCG GCC GAG ACA ATG TAC
     339
```

```
Ile Phe Arg Tyr Cys Ser Gly Ser Cys Glu Ala Ala Glu Thr Met Tyr
     90                  95                 100

GAC AAA ATA CTA AAA AAT CTG TCT CGA AGT AGA AGG CTA ACA AGT GAC
     387
Asp Lys Ile Leu Lys Asn Leu Ser Arg Ser Arg Arg Leu Thr Ser Asp
105                 110                 115                 120

AAG GTA GGC CAG GCA TGT TGC AGG CCG GTC GCC TTC GAC GAC GAC CTG
     435
Lys Val Gly Gln Ala Cys Cys Arg Pro Val Ala Phe Asp Asp Asp Leu
               125                 130                 135

TCG TTT TTA GAC GAC AGC CTG GTT TAC CAT ATC CTA AGA AAG CAT TCC
     483
Ser Phe Leu Asp Asp Ser Leu Val Tyr His Ile Leu Arg Lys His Ser
            140                 145                 150

GCT AAA CGG TGT GGA TGT ATC TGA CCC TGG CTC CAG AGA
      522
Ala Lys Arg Cys Gly Cys Ile
       155
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 159 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Met Pro Glu Asp Tyr Pro Asp Gln Phe Asp Asp Val Met Asp Phe Ile
  1               5                  10                  15

Gln Ala Thr Ile Lys Arg Leu Lys Arg Ser Pro Asp Lys Gln Ala Ala
            20                  25                  30

Ala Leu Pro Arg Arg Glu Arg Asn Arg Gln Ala Ala Ala Ala Ser Pro
        35                  40                  45

Glu Asn Ser Arg Gly Lys Gly Arg Arg Gly Gln Arg Gly Lys Asn Arg
    50                  55                  60
```

```
Gly Cys Val Leu Thr Ala Ile His Leu Asn Val Thr Asp Leu Gly Leu
 65                  70                  75                  80

Gly Tyr Glu Thr Lys Glu Glu Leu Ile Phe Arg Tyr Cys Ser Gly Ser
                 85                  90                  95


Cys Glu Ala Ala Glu Thr Met Tyr Asp Lys Ile Leu Lys Asn Leu Ser
            100                 105                 110


Arg Ser Arg Arg Leu Thr Ser Asp Lys Val Gly Gln Ala Cys Cys Arg
        115                 120                 125


Pro Val Ala Phe Asp Asp Asp Leu Ser Phe Leu Asp Asp Ser Leu Val
    130                 135                 140


Tyr His Ile Leu Arg Lys His Ser Ala Lys Arg Cys Gly Cys Ile
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
GTCTACGGAG ACCGGATCCG
      20
```

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
CTCTGGAGCC AGGGTCAGAT AC
      22
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TCCCGCTGCC CGCCGCAAAT ATGCCAGAGG AT
32

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ACCGCGTCCG CCTTCCCGCT GCCAACCCAG AGA
33

What Is Claimed Is:

1. An isolated astrocyte derived neurotrophic factor (ATF) protein.

2. The ATF protein according to claim 1 comprising ATF-1, ATF-2, or ATF-3.

3. The ATF protein according to claim 2 having an amino acid sequence selected from the group consisting of sequences set forth in SEQ ID NO:2, SEQ ID NO:31, SEQ ID NO:33, and SEQ ID NO:37.

4. A pharmaceutical composition comprising the protein according to claim 1 and a pharmaceutically acceptable carrier.

5. An isolated nucleic acid molecule which encodes the protein according to claim 1.

6. An isolated nucleic acid molecule which encodes the protein according to claim 2.

7. The isolated nucleic acid molecule of claim 5, wherein said nucleic acid molecule has a sequence selected from the group consisting of sequences set forth in SEQ ID NO:1, SEQ ID NO:30, SEQ ID NO:32, and SEQ ID NO:36.

8. A recombinant nucleic acid molecule comprising, 5′ to 3′, a promoter effective to initiate transcription in a host cell and the isolated nucleic acid molecule according to claim 5.

9. A recombinant nucleic acid molecule comprising a vector and the isolated nucleic acid molecule according to claim 5.

10. A cell that contains the recombinant nucleic acid molecule according to claim 8 or 9.

11. A method for increasing the survival of a cell comprising the step of contacting said cell with the protein of claim 1.

12. The method of claim 11 wherein said cell is a dopaminergic neuron.

13. An isolated nucleic acid molecule consisting essentially of a nucleotide sequence selected from the group consisting of sequences set forth in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:28 and SEQ ID NO:29, wherein said nucleotide sequence is expressed at a higher level in T1 astrocytes compared to the level expressed in T2 astrocytes.

14. An isolated protein comprising an amino acid sequence encoded by the nucleic acid molecule of claim 13.

15. A recombinant nucleic acid molecule comprising a vector and the isolated nucleic acid molecule according to claim 13.

16. A cell that contains the recombinant nucleic acid molecule according to claim 15.

17. An isolated nucleic acid molecule consisting essentially of a nucleotide sequence selected from the group consisting of sequences set forth in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.

18. An isolated protein comprising an amino acid sequence encoded by the nucleic acid molecule of claim 17.

19. A recombinant nucleic acid molecule comprising a vector and the isolated nucleic acid molecule according to claim 17.

20. A cell that contains the recombinant nucleic acid molecule according to claim 19.

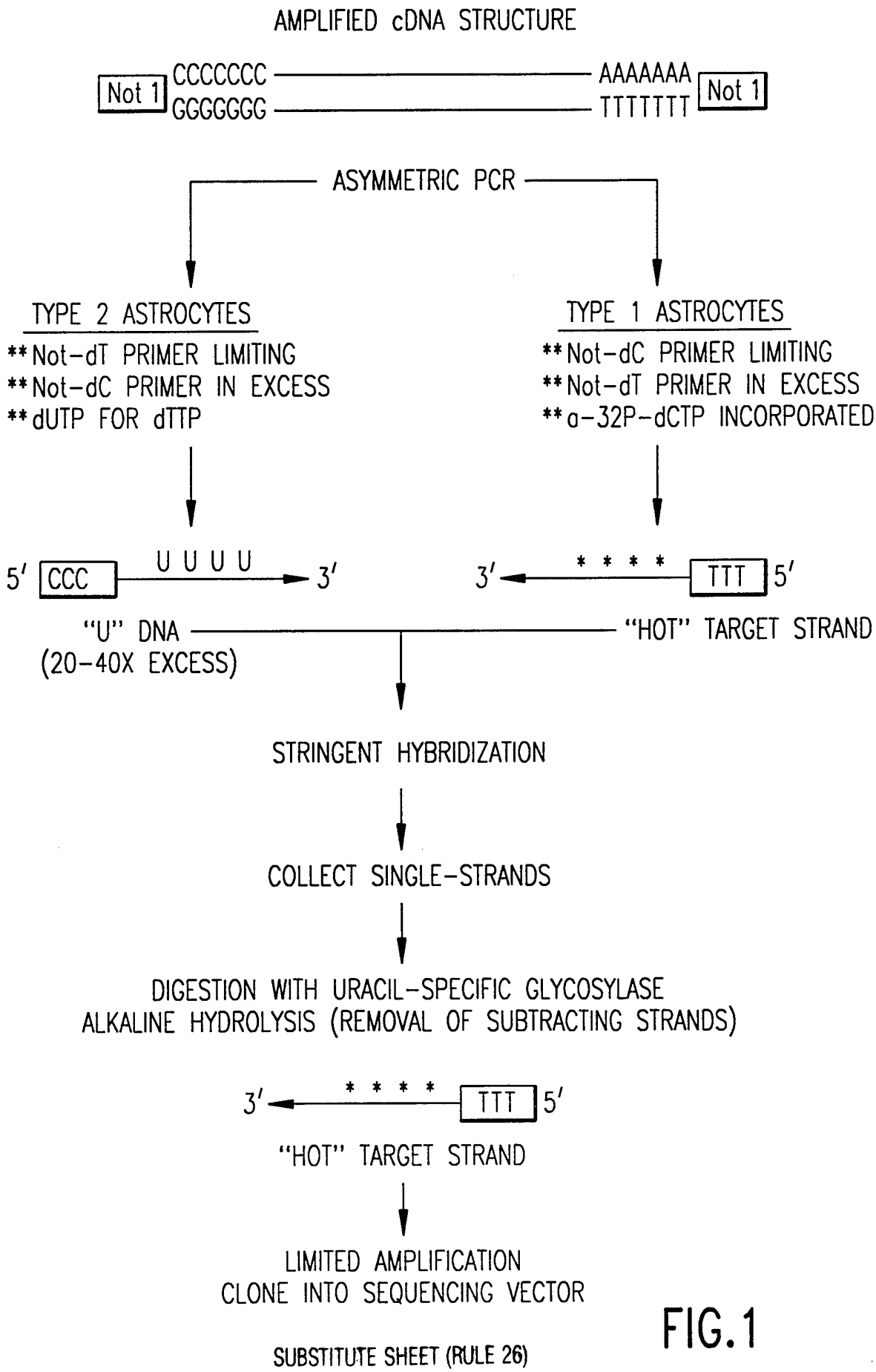
AMPLIFIED cDNA STRUCTURE
Not 1
CCCCCCC
GGGGGGG
AAAAAAA
TTTTTTT
Not 1
ASYMMETRIC PCR
TYPE 2 ASTROCYTES
**Not-dT PRIMER LIMITING
**Not-dC PRIMER IN EXCESS
**dUTP FOR dTTP
TYPE 1 ASTROCYTES
**Not-dC PRIMER LIMITING
**Not-dT PRIMER IN EXCESS
**α-32P-dCTP INCORPORATED
5′ CCC U U U U 3′
3′ * * * * TTT 5′
"U" DNA
(20-40X EXCESS)
"HOT" TARGET STRAND
STRINGENT HYBRIDIZATION
COLLECT SINGLE-STRANDS
DIGESTION WITH URACIL-SPECIFIC GLYCOSYLASE
ALKALINE HYDROLYSIS (REMOVAL OF SUBTRACTING STRANDS)
3′ * * * * TTT 5′
"HOT" TARGET STRAND
LIMITED AMPLIFICATION
CLONE INTO SEQUENCING VECTOR

FIG.1

```
  1 GGTCTACGGAGACCGGATCCGAGGTGCCGCCGCCGGACGGGACTCTAAGA  50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 GGTCTACGGAGACCGGATCCGAGGTGCCGCCGCCGGACGGGACTCTAAGA  50

 51 TGAAGTTATGGGATGTCGTGGCTGTCTGCCTGGTGTTGCTCCACACCGCG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 TGAAGTTATGGGATGTCGTGGCTGTCTGCCTGGTGTTGCTCCACACCGCG 100

101 TCTGCCTTCCCGCTGCCCGCCG..........................  122
    |||||||||||||||||||||||
101 TCTGCCTTCCCGCTGCCCGCCGGTAAGAGGCTTCTCGAAGCGCCCGCCGA 150

123 CCAATATGCCCGAAGATTATCCTGACCAGTTTGATGACGTCATGGATTTT 172
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 CCAATATGCCCGAAGATTATCCTGACCAGTTTGATGACGTCATGGATTTT 250

173 ATTCAAGCCACCATCAAAAGACTGAAAAGGTCACCAGATAAACAAGCGGC 222
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 ATTCAAGCCACCATCAAAAGACTGAAAAGGTCACCAGATAAACAAGCGGC 300

223 GGCACTTCCTCGAAGAGAGAGGAACCGGCAAGCTGCAGCTGCCAGCCCAG 272
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 GGCACTTCCTCGAAGAGAGAGGAACCGGCAAGCTGCAGCTGCCAGCCCAG 350

273 AGAATTCCAGAGGGAAAGGTCGCAGAGGCCAGAGGGGCAAAAATCGGGGG 322
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 AGAATTCCAGAGGGAAAGGTCGCAGAGGCCAGAGGGGCAAAAATCGGGGG 400

323 TGCGTCTTAACTGCAATACACTTAAATGTCACTGACTTGGGTTTGGGCTA 372
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 TGCGTCTTAACTGCAATACACTTAAATGTCACTGACTTGGGTTTGGGCTA 450

373 CGAAACCAAGGAGGAACTGATCTTTCGATATTGTAGCGGTTCCTGTGAAG 422
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 CGAAACCAAGGAGGAACTGATCTTTCGATATTGTAGCGGTTCCTGTGAAG 500
```

FIG.2A

```
423  CGGCCGAGACAATGTACGACAAAATACTAAAAAATCTGTCTCGAAGTAGA  472
     ||||||||||||||||||||||||||||||||||||||||||||||||||
501  CGGCCGAGACAATGTACGACAAAATACTAAAAAATCTGTCTCGAAGTAGA  550

473  AGGCTAACAAGTGACAAGGTAGGCCAGGCATGTTGCAGGCCGGTCGCCTT  522
     ||||||||||||||||||||||||||||||||||||||||||||||||||
551  AGGCTAACAAGTGACAAGGTAGGCCAGGCATGTTGCAGGCCGGTCGCCTT  600

523  CGACGACGACCTGTCGTTTTTAGACGACAGCCTGGTTTACCATATCCTAA  572
     ||||||||||||||||||||||||||||||||||||||||||||||||||
601  CGACGACGACCTGTCGTTTTTAGACGACAGCCTGGTTTACCATATCCTAA  650

573  GAAAGCATTCCGCTAAACGGTGTGGATGTATCTGACCCTGGCTCCAGAGA  622
     ||||||||||||||||||||||||||||||||||||||||||||||||||
651  GAAAGCATTCCGCTAAACGGTGTGGATGTATCTGACCCTGGCTCCAGAGA  700
```

FIG.2B

```
                .         .         .         .         .
  1 MKLWDVVAVCLVLLHTASAFPLPAA.......................... 25
    |||||||||||||||||||||||||:
  1 MKLWDVVAVCLVLLHTASAFPLPAGKRLLEAPAEDHSLGHRRVPFALTSD 50
                .         .         .         .         .
 26 .NMPEDYPDQFDDVMDFIQATIKRLKRSPDKQAAALPRRERNRQAAAASP 74
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 SNMPEDYPDQFDDVMDFIQATIKRLKRSPDKQAAALPRRERNRQAAAASP 100
                .         .         .         .         .
 75 ENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELIFRYCSGSCE 124
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 ENSRGKGRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELIFRYCSGSCE 150
                .         .         .         .         .
125 AAETMYDKILKNLSRSRRLTSDKVGQACCRPVAFDDDLSFLDDSLVYHIL 174
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 AAETMYDKILKNLSRSRRLTSDKVGQACCRPVAFDDDLSFLDDSLVYHIL 200
                .
175 RKHSAKRCGCI  185
    ||||||||||||
201 RKHSAKRCGCI  211
```

FIG.3

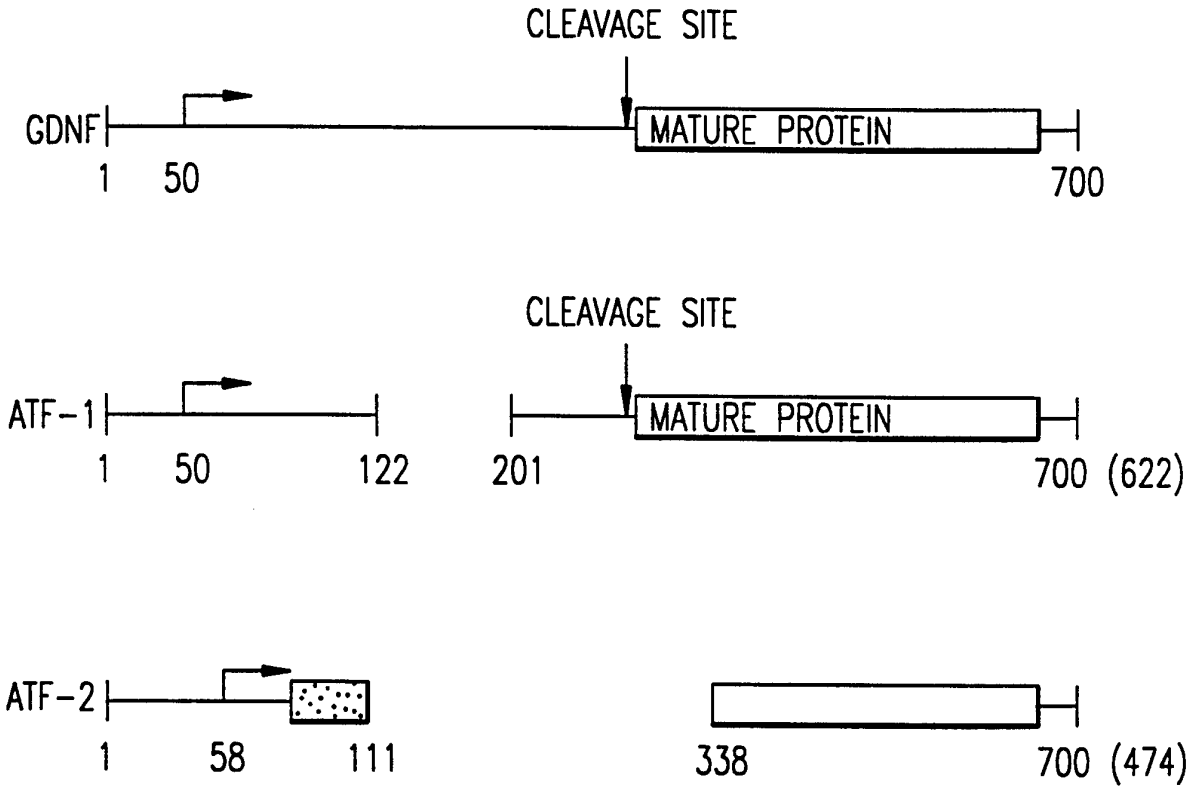
CLEAVAGE SITE
GDNF
MATURE PROTEIN
1
50
700
CLEAVAGE SITE
ATF-1
MATURE PROTEIN
1
50
122
201
700 (622)
ATF-2
1
58
111
338
700 (474)

FIG.4

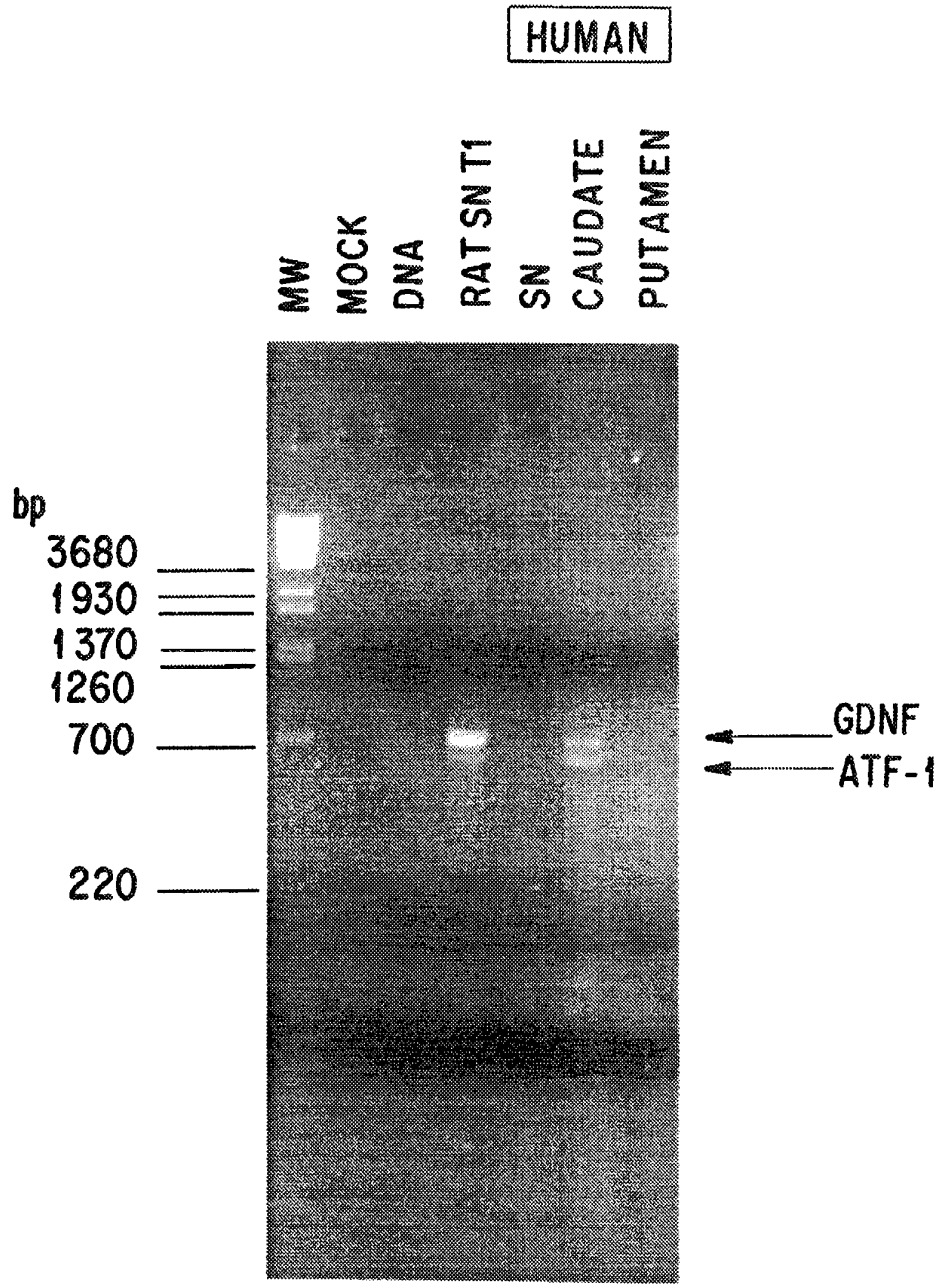
HUMAN
MW
MOCK DNA
RAT SN T1
SN CAUDATE
PUTAMEN
bp
3680
1930
1370
1260
700
220
GDNF
ATF-1

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14771

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.4, 70.1, 70.3, 71.1, 172.1, 240.1, 240.2, 243, 320.1; 514/2, 12; 530/350; 536/23.1, 23.5, 23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 260, issued 21 May 1993, L. Lin et al, "GDNF: A Glial Cell Line-Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons", pages 1130-1132, see especially page 1131.	1-12; 17-20
Y	WO 93/06116 (LIN ET AL), issued 01 April 1993, see entire document.	1-12
A	S. MURPHY, ed., "ASTROCYTES: PHARMACOLOGY AND FUNCTION" published 1993 by Academic Press (San Diego, CA), pages 267-305.	1-20
Y	J. Neurosci. Res., Volume 27, Number 2, issued October 1990, T. Rhyner et al, "Isolation of cDNAs From a Mouse Astroglial Cell Line by a Subtracted cDNA Library", pages 144-152, see entire document.	13-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 APRIL 1995	12APR1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ARIE M. MICHELSOHN, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14771

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Society for Neuroscience Abstracts, Volume 17, issued 1991, A. Gard et al, "Oligodendrocyte Survival Selectively Determined by a Soluble Astrocyte-Derived Factor", page 46, see entire document.	1-12
Y	Society for Neuroscience Abstracts, Volume 19, issued November 1993, J. Fredieu et al, "Astrocyte Specific cDNAs Isolated by Subtraction Hybridization", page 59, see entire document.	13-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14771

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): -

A61K 38/00, 38/16, 38/18, 38/22; C07H 21/00, 21/04; C07K 14/00, 14/435, 14/475; 14/575; C12N 5/10, 5/16, 7/01,15/00,15/09, 15/10, 15/11, 15/12, 15/16, 15/18, 15/63; C12P 21/00, 21/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.4, 70.1, 70.3, 71.1, 172.1, 240.1, 240.2, 243, 320.1; 514/2, 12; 530/350; 536/23.1, 23.5, 23.51

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; STN files: biosis, medline, EMBASE, ca, wpids; SEARCH TERMS gdnf (glial dervied neurotrophic factor#), astrocyte-derived(2a)factor, astrocyte#/cDNA(clon?)/subtract?(w)hybridization; TGF.beta? glia or oligodendrocyte# or astrocyte# (in various combinations with previous terms); electronic sequence search.