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(57) Abstract

A human F-spondin-like protein and DNA (RNA) encoding such protein and a procedure for producing such protein by recombinant techniques is disclosed. Also disclosed are methods for utilizing such polypeptide for treating spinal cord injuries and damage to peripheral nerves by promoting neural-cell adhesion and neurite extension, inhibiting tumor metastases and tumor angiogenesis, and stimulating wound repair. Antagonists are also disclosed which may be utilized to prevent malaria. Diagnostic assays for identifying mutations in nucleic acid sequence encoding a polypeptide of the present invention and for detecting altered levels of the polypeptide of the present invention for detecting diseases, for example, cancer, are also disclosed.

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HUMAN NEURONAL ATTACHMENT FACTOR-1

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention has been putatively identified as a human neuronal attachment factor-1, sometimes hereinafter referred to as "NAF-1". The invention also relates to inhibiting the action of such polypeptides.

F-spondin (FSP) is a gene that is predominantly expressed during the early development of the vertebrate nervous system. The main function is thought to be in neural cell pattern formation and axonal growth. It was found in a subtractive hybridization screen designed to isolate floor-plate specific genes. The floor-plate provides diffusible signals that act on the neurons that extend from the developing spinal cord. These signals can lead to chemoattraction and fasciculation of commissural axons in the ventral midline. F-spondin mRNA is expressed at high levels in the developing neural tube at the ventral midline even before cell differentiation markers can detect the floor- plate. F-spondin is not detectable in other regions of the spinal cord until later in embryonic life. There is also transient F-spondin expression early in

peripheral nerve development which diminishes to undetectable levels following birth. The adult central nervous system contains F-spondin while the peripheral nerve (sciatic nerve) does not. Outside the adult nervous system, organs such as the lung and kidney also express F-spondin. The protein is 807 amino acids and codes for a predicted 90 kD polypeptide. The apparent size is approximately 116 kD by SDS-PAGE which indicates post-translational modifications such as glycosylation. There are six domains homologous to the thrombospondin (TSP) type 1 repeats (TSR) which have been shown to control cell adhesion. The protein has been expressed in COS cells and purified as a myc-tag fusion protein. This protein was active in promoting neurite extension and adhesion of embryonic dorsal root ganglion and dorsal spinal cords respectively. It was not chemotropic for embryonic dorsal spinal cord neurons. (Klar, A. et al., Cell, 69:95-110 (1992)).

The C-terminal half of F-spondin contains 6 repeats identified in thrombospondin and other proteins implicated in cell adhesion. Thrombospondin is a 450,000-dalton glyco-protein secreted by platelets in response to such physiological activators as thrombin and collagen (Lawler, J., Blood, 67:1197-1209 (1986)). TSP comprises 3% of the total platelet protein and 25% of the total platelet-secreted proteins (Tuszynski, G.P., et al., J. Biol. Chem., 260:12240-12245 (1985)). Although the precise biological role of TSP has yet to be fully established, it is generally accepted that TSP plays a major role in cell adhesion and cell-cell interactions. It should be pointed out that the C-terminal repeats present in thrombospondin may have different biological activities.

TSP was found to promote the cell-substratum adhesion of a variety of cells, including platelets, melanoma cells, smooth muscle cells, endothelial cells, fibroblasts and epithelial cells (Tuszynski, G.P., et al., Science (Washington, DC), 236:1570-1573 (1983)).

Thrombospondin has been postulated to play a role in malarial infection induced by only one strain of malaria, *plasmodium falciparum*. During malarial infection, TSP promotes adhesion of parasitized red cells to endothelial cells (Roberts, D. D., et al., Nature (Lond.), 318:64-66 (1984)) and during tumor cell metastases TSP promotes adhesion of mouse sarcoma cells to the vascular bed and expression of the malignant phenotype of small cell carcinoma (Castle, V. J., J. Clin. Invest., 87:1883-1883 (1991)).

Properdin is a complement-binding protein which also contains the 6 terminal repeats found in thrombospondin. UNC-5, a *C. elegans* gene that bears two terminal repeats, appears to guide the axonal extension of the sub-set of neurons. These proteins, which contain at least one member of the six terminal repeats, form a family of proteins which have related functions.

The gene and polypeptide encoded thereby of the present invention has been putatively identified as an Neuronal Attachment Factor-1 protein as a result of amino acid sequence homology to rat F-spondin.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptide of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding a polypeptide of the present invention including mRNAs, cDNAs, genomic DNAs as well as analogs and biologically active and diagnostically or therapeutically useful fragments thereof.

In accordance with another aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97343.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention, under conditions promoting expression of said protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, to treat spinal cord injuries or damage to peripheral nerves by promoting neural cell adhesion and neurite extension, to inhibit tumor cell metastases, inhibit endothelial cell proliferation, adhesion and motility, to decrease tumor neovascularization, to be angiostatic for tumor cells and to promote wound healing.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides, which would bind to and neutralize NAF-1 to inhibit its putative cell adhesion properties to restrict metastases, particularly tumor metastases.

In accordance with another aspect of the present invention, there are provided NAF-1 agonists which mimic NAF-1 and binds to the NAF-1 receptors.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, in the treatment of malarial infection caused by *Plasmodium falciparum*.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to hybridize to a nucleic acid sequence of the present invention.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for

detecting diseases or susceptibility to diseases related to mutations in the nucleic acid sequences encoding a polypeptide of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for *in vitro* purposes related to scientific research, for example, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 is an illustration of the cDNA and corresponding amino acid sequence of the polypeptide of the present invention. Sequencing was performed using a 373 automated DNA sequencer (Applied Biosystems, Inc.).

Figure 2 is an amino acid sequence comparison between the polypeptide of the present invention (bottom line) and rat F-spondin (rFSP) (top line) (SEQ ID NO:7).

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2).

The polynucleotide of this invention was discovered in a cDNA library derived from human epithelioid sarcoma. It is structurally related to the rat F-spondin family. It contains an open reading frame encoding a protein of 331 amino acid residues. The protein exhibits the highest degree of homology to rat F-spondin with 33.1% identity and 52.9% similarity over the entire amino acid stretch. The gene of the present invention shows the greatest homology at the nucleotide level to the rat F-spondin gene with 66% similarity and 66% identity. It is also important that the polypeptide of the present invention contains the conserved

motif, WSXW, which is a potential binding sequence for polypeptides in this family.

Northern blot analysis of the protein of the present invention showed a broad band at 1.6-1.9 kb in liver and lower level expression in kidney, lung, heart and placenta. Brain expression was barely detectable. Two libraries which were constructed from tissues induced to undergo apoptosis, apoptotic T-cells (HTG) and TNF induced amniotic cells (HAU), had one clone in each. By extrapolation, NAF-1 was represented at least 50 times more frequently in apoptotic t-cells expressed sequence tags than all normal and activated t-cell libraries. In the TNF induced amniotic cells library, NAF-1 was detected 1 out of 2,414 expressed sequence tags versus 0 out of 3,595 expressed sequence tags for the non-TNF treated amniotic cell library.

The NAF-1 cDNA contains an open reading frame encoding a polypeptide of 35.8 kD. Amino acids 1-23 encode a putative signal peptide. NAF-1 also contains a putative N-linked glycosylation site at position 303. The homology of NAF-1 to FSP covers amino acids 199-495 of the latter protein. Thus, NAF-1 does not appear to be the human counterpart of the rat FSP. NAF-1 contains only one TSR which begins at amino acid 278. This region is much more homologous to FSP type 1 repeats than to those of TSP, 38% versus 20%, respectively. The amino terminal 277 amino acids of NAF-1 share homology to FSP but show no resemblance to any other known proteins.

In accordance with another aspect of the present invention there are provided isolated polynucleotides encoding a mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97343, deposited with the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, USA, on November 20, 1995. The deposited material is a pBluescript SK (-) (Stratagene, La Jolla, CA) plasmid that contains the full-length NAF-1 cDNA. The NAF-1 cDNA has been cloned into the EcoRI, XhoI site.

The deposit has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. The strain will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposit is provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotide contained in the deposited material, as well as the amino acid sequence of the polypeptides encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited material, and no such license is hereby granted.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1).

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID NO:2) may include, but is not limited to: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide

which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID NO:2) as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence

is cleaved an active mature protein remains. Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, at least 50 bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to

determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1).

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 and polynucleotides complementary thereto as well as portions thereof, which portions have at least 30 consecutive bases and preferably at least 50 consecutive bases and to polypeptides encoded by such polynucleotides.

The present invention further relates to a polypeptide which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2), as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2), means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all

of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the

form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTRC99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers

are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

NAF-1 may be employed to treat spinal cord injuries or damage to peripheral nerves by increasing spinal cord and sensory neuron attachment and neurite outgrowth.

NAF-1 may also be employed to inhibit tumor cell metastases induced by small cell carcinoma. The NAF-1 gene and gene product of the present invention may also be employed to reduce primary tumor growth, metastatic potential and angiogenesis in human breast carcinoma cells.

The NAF-1 gene and gene product of the present invention may also be employed to promote wound healing due to its ability to promote cell-cell interaction and cell adhesion.

NAF-1 may also be employed to modulate hemostasis.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to human disease.

This invention provides a method for identification of the receptor for NAF-1. The gene encoding methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to NAF-1, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to NAF-1. Transfected cells which are grown on glass slides are exposed to labeled NAF-1 ligand. NAF-1 can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to auto-radiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually yielding a single clone that encodes the putative receptor. As an alternative approach for receptor identification, labeled ligand can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the ligand-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention provides a method of screening compounds to identify those which are agonists to or antagonists to NAF-1. The identification of both type compounds would involve a neurite outgrowth assay. COS cells (5×10^8) are transfected with NAF-1/pcDNA-1 (Invitrogen, Inc.) and conditioned medium is collected. NAF^{myc} is affinity purified on a monoclonal anti-myc (9E10) affinity-purified F-$spondin^{myc}$ (20 μg/ml) is absorbed onto nitrocellulose (Lemmon et al., 1989). For controls,

parental COS cell-conditioned medium is purified on the same column and used as a substrate on nitrocellulose. The nitrocellulose is then blocked with BSA (10 mg/ml), which provided a further control for background neurite outgrowth. rAT E14 DRG neurons are plated on immobilized protein substrates at a density of 2-10 x 10^4 cells per 35 mm tissue culture dish (Nunc) and grown for 14 hr. Cultures are then fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained using MAb 3A10 (Furley et al., 1990; available from Developmental Studies Hybridoma Bank), which recognizes a neuronal filament-associated protein and serves as a marker for fine neurites. Neuronal cell bodies and neurites are visualized by indirect immunofluorescence on a Zeiss Axioplan microscope. Neurite lengths are measured as the distance from the edge of the soma (sharply defined by 3A10 fluorescence) to the tip of its longest neurite. Neurite lengths are measured if the entire length of the neurite could be unambiguously identified. About 25 neurites are measurable within each protein-coated area (3-4mm^2).

Rat e13 dorsal spinal cord neurons can also be assayed by plating the dissociated cells on immobilized protein substrate at a density of 10^6 cells per 35 mm tissue culture dish (Nunc). After 1 hr. the cultures are washed twice with PBS and fixed in 4% paraformaldehyde. Cells are counted on a Zeiss Axioplan microscope at 400 x magnification. Ten independent counts are taken from each experiment.

An alternative example of identifying agonists and antagonists to the polypeptide of the present invention includes expressing the NAF-1 receptor from a mammalian cell or membrane preparation and incubating that receptor with labeled NAF-1 in the presence of a compound. The ability of a compound to enhance or block the interaction is then quantified. Alternatively, the response of a known second messenger system following interaction of NAF-1 and its receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems

include, but are not limited, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

Potential antagonists include an antibody, or in some cases, an oligopeptide, which binds to the polypeptide. Alternatively, a potential antagonist may be a closely related protein which binds to the receptor sites, however, they are inactive forms of the polypeptide and thereby prevent the action of NAF-1 since receptor sites are occupied.

Another potential antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of NAF-1. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into NAF-1 polypeptide (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of NAF-1.

Potential antagonists include a small molecule which binds to and occupies the catalytic site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to treat malarial infection induced by *Plasmodium falciparum*. During malarial infection, the polypeptide of the present invention may promote adhesion of parasitized red cells to endothelial cells and, therefore, antagonists would inhibit this action and prevent malaria.

The polypeptides of the present invention or antagonists and agonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or antagonists or agonist, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides of the present invention or agonists or antagonists may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, parenterally, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10 μg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg

body weight daily, taking into account the routes of administration, symptoms, etc.

The NAF-1 polypeptides and agonists and antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art and are apparent from the teachings herein. For example, cells may be engineered by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. For example, a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such

means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the gene of the present invention as a diagnostic. Detection of a mutated form of the gene will allow a diagnosis of a disease or a susceptibility to a disease which results from underexpression of NAF-1, for example, tumor metastases and tumor angiogenesis.

Individuals carrying mutations in the gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, including but not limited to blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding NAF-1 can be used to identify and analyze mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to

radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between the reference gene and genes having mutations may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of the polypeptide of the present invention in various tissues since an over-expression of the proteins compared to normal control tissue samples can detect the presence of NAF-1 and conditions related to an overexpression of NAF-1, for example, tumor metastases and angiogenesis. Assays used to detect levels of the polypeptide of the present invention in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis and preferably an ELISA assay. An ELISA assay initially comprises preparing an antibody specific to the NAF-1 antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attached to any of the polypeptide of the present invention attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to the polypeptide of the present invention. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of the polypeptide of the present invention present in a given

volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to the polypeptide of the present invention are attached to a solid support and labeled NAF-1 and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of the polypeptide of the present invention in the sample.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its

chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA having at least 50 or 60 bases. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well

as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptide of the present invention by attachment of the antibody to a solid support for isolation and/or purification by affinity chromatography.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to

such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5'

phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

Example 1

Bacterial Expression and Purification of NAF-1

The DNA sequence encoding NAF-1, ATCC # 97343, is initially amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed NAF-1 protein (minus the signal peptide sequence) and the vector sequences 3' to the NAF-1 gene. Additional nucleotides corresponding to NAF-1 are added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' GCCATACG**GGATCC**CAGCCTCTTGGGGGAGAGTCC 3' (SEQ ID NO:3) contains a BamHI restriction enzyme site followed by 21 nucleotides of NAF-1 coding sequence starting from the presumed terminal amino acid of the processed protein codon. The 3' sequence 5' GGCATACG**TCTAGA**TTAGACGCAGTTATCAGGGAC 3' (SEQ ID NO:4) contains complementary sequences to an XbaI site and is followed by 21 nucleotides of NAF-1. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-

9 is then digested with BamHI and XbaI. The amplified sequences are ligated into pQE-9 and are inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture is then used to transform E. coli strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 ($O.D._{600}$) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized NAF-1 is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag. NAF-1 is eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein is dialyzed to 10 mmolar sodium phosphate.

Example 2

Cloning and expression of NAF-1 using the baculovirus expression system

The DNA sequence encoding the full length NAF-1 protein, ATCC # 97343, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCCATACG**GGATCC**GCC ATCATGGAAAACCCCAGCCCGGCC 3' (SEQ ID NO:5) and contains a BamHI restriction enzyme site (in bold) followed by 8 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (Kozak, M., J. Mol. Biol., 196:947-950 (1987) which is just behind the first 21 nucleotides of the NAF-1 gene (the initiation codon for translation "ATG" is underlined).

The 3' primer has the sequence 5' GGCATACG**TCTAGA**TTA GACGCAGTTATCAGGGAC 3' (SEQ ID NO:6) and contains the cleavage site for the restriction endonuclease XbaI and 21 nucleotides complementary to the 3' end of the translated sequence of the NAF-1 gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases BamHI and XbaI and then purified again on a 1% agarose gel. This fragment is designated F2.

The vector pRG1 (modification of pVL941 vector, discussed below) is used for the expression of the NAF-1 protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases BamHI and XbaI. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the

polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E.coli XL1 blue cells were then transformed and bacteria identified that contained the plasmid (pBacNAF-1) with the NAF-1 gene using the enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 μg of the plasmid pBacNAF-1 was co-transfected with 1.0 μg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBacNAF-1 were mixed in a sterile well of a microtiter plate containing 50 μl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 μl Lipofectin plus 90 μl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10%

fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution the virus was added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-NAF-1 at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Example 3

Expression of Recombinant NAF-1 in COS cells

Expression of plasmid, NAF-1 HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli

replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire NAF-1 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, placing the recombinant protein expression under control of the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The fusion of HA tag to the NAF-1 protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding NAF-1, ATCC # 97343, is constructed by PCR using two primers as described in the above examples. The 5' primer contains a convenient restriction site followed by a portion of NAF-1 coding sequence starting from the initiation codon; the 3' sequence contains complementary sequences to a convenient restriction site, translation stop codon, HA tag and the last several nucleotides of the NAF-1 coding sequence (not including the stop codon). Therefore, the PCR product contains a convenient 5' and 3' restriction sites, NAF-1 coding sequence followed by HA tag fused in frame, and a translation termination stop codon next to the HA tag. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested and ligated. The ligation mixture is transformed into E. coli strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant NAF-1, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular

Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the NAF-1 HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with ^{35}S-cysteine two days post transfection. Culture media is then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media are precipitated with an HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

Example 4

Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hastings, Gregg

(ii) TITLE OF INVENTION: Human Neuronal Attachment Factor-1

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein
(B) STREET: 6 Becker Farm Road
(C) CITY: Roseland
(D) STATE: NJ
(E) COUNTRY: USA
(F) ZIP: 07068-1739

(v) 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 #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ferraro, Gregory D
(B) REGISTRATION NUMBER: 36,134
(C) REFERENCE/DOCKET NUMBER: 325800-491

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-994-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1105 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 19..87

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 88..1011

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 19..1011

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

```
CGCTGCTCCT GCCGGGTG ATG GAA AAC CCC AGC CCG GCC GCC GCC CTG GGC          51
                    Met Glu Asn Pro Ser Pro Ala Ala Ala Leu Gly
                    -23         -20                 -15

AAG GCC CTC TGC GCT CTC CTC CTG GCC ACT CTC GGC GCC GCC GGC CAG          99
Lys Ala Leu Cys Ala Leu Leu Leu Ala Thr Leu Gly Ala Ala Gly Gln
        -10                 -5                  1

CCT CTT GGG GGA GAG TCC ATC TGT TCC GCC AGA GCC CTG GCC AAA TAC         147
Pro Leu Gly Gly Glu Ser Ile Cys Ser Ala Arg Ala Leu Ala Lys Tyr
  5                 10                  15                  20

AGC ATC ACC TTC ACG GGC AAG TGG AGC CAG ACG GCC TTC CCC AAG CAG         195
Ser Ile Thr Phe Thr Gly Lys Trp Ser Gln Thr Ala Phe Pro Lys Gln
                25                  30                  35

TAC CCC CTG TTC CGC CCC CCT GCG CAG TGG TCT TCG CTG CTG GGG GCC         243
Tyr Pro Leu Phe Arg Pro Pro Ala Gln Trp Ser Ser Leu Leu Gly Ala
            40                  45                  50

GCG CAT AGC TCC GAC TAC AGC ATG TGG AGG AAG AAC CAG TAC GTC AGT         291
Ala His Ser Ser Asp Tyr Ser Met Trp Arg Lys Asn Gln Tyr Val Ser
        55                  60                  65

AAC GGG CTG CGC GAC TTT GCG GAG CGC GGC GAG GCC TGG GCG CTG ATG         339
Asn Gly Leu Arg Asp Phe Ala Glu Arg Gly Glu Ala Trp Ala Leu Met
    70                  75                  80

AAG GAG ATC GAG GCG GCG GGG GAG GCG CTG CAG AGC GTG CAC GCG GTG         387
Lys Glu Ile Glu Ala Ala Gly Glu Ala Leu Gln Ser Val His Ala Val
85                  90                  95                  100

TTT TCG GCG CCC GCC GTC CCC AGC GGC ACC GGG CAG ACG TCG GCG GAG         435
Phe Ser Ala Pro Ala Val Pro Ser Gly Thr Gly Gln Thr Ser Ala Glu
                105                 110                 115

CTG GAG GTG CAG CGC AGG CAC TCG CTG GTC TCG TTT GTG GTG CGC ATC         483
Leu Glu Val Gln Arg Arg His Ser Leu Val Ser Phe Val Val Arg Ile
            120                 125                 130

GTG CCC AGC CCC GAC TGG TTC GTG GGC GTG GAC AGC CTG GAC CTG TGC         531
Val Pro Ser Pro Asp Trp Phe Val Gly Val Asp Ser Leu Asp Leu Cys
        135                 140                 145

GAC GGG GAC CGT TGG CGG GAA CAG GCG GCG CTG GAC CTG TAC CCC TAC         579
Asp Gly Asp Arg Trp Arg Glu Gln Ala Ala Leu Asp Leu Tyr Pro Tyr
    150                 155                 160

GAC GCC GGG ACG GAC AGC GGC TTC ACC TTC TCC TCC CCC AAC TTC GCC         627
Asp Ala Gly Thr Asp Ser Gly Phe Thr Phe Ser Ser Pro Asn Phe Ala
165                 170                 175                 180

ACC ATC CCG CAG GAC ACG GTG ACC GAG ATA ACG TCC TCC TCT CCC AGC         675
Thr Ile Pro Gln Asp Thr Val Thr Glu Ile Thr Ser Ser Ser Pro Ser
                185                 190                 195

CAC CCG GCC AAC TCC TTC TAC TAC CCG CGG CTG AAG GCC CTG CCT CCC         723
His Pro Ala Asn Ser Phe Tyr Tyr Pro Arg Leu Lys Ala Leu Pro Pro
            200                 205                 210

ATC GCC AGG GTG ACA CTG GTG CGG CTG CGA CAG AGC CCC AGG GCC TTC         771
Ile Ala Arg Val Thr Leu Val Arg Leu Arg Gln Ser Pro Arg Ala Phe
        215                 220                 225
```

```
ATC CCT CCC GCC CCA GTC CTG CCC AGC AGG GAC AAT GAG ATT GTA GAC        819
Ile Pro Pro Ala Pro Val Leu Pro Ser Arg Asp Asn Glu Ile Val Asp
    230                 235                 240

AGC GCC TCA GTT CCA GAA ACG CCG CTG GAC TGC GAG GTC TCC CTG TGG        867
Ser Ala Ser Val Pro Glu Thr Pro Leu Asp Cys Glu Val Ser Leu Trp
245                 250                 255                 260

TCG TCC TGG GGA CTG TGC GGA GGC CAC TGT GGG AGG CTC GGG ACC AAG        915
Ser Ser Trp Gly Leu Cys Gly Gly His Cys Gly Arg Leu Gly Thr Lys
                265                 270                 275

AGC AGG ACT CGC TAC GTC CGG GTC CAG CCC GCC AAC AAC GGG AGC CCC        963
Ser Arg Thr Arg Tyr Val Arg Val Gln Pro Ala Asn Asn Gly Ser Pro
            280                 285                 290

TGC CCC GAG CTC GAA GAA GAG GCT GAG TGC GTC CCT GAT AAC TGC GTC       1011
Cys Pro Glu Leu Glu Glu Glu Ala Glu Cys Val Pro Asp Asn Cys Val
        295                 300                 305

TAAGACCAGA GCCCCGCAGC CCCTGGGGCC CCCCGGAGCC ATGGGGTGTC GGGGGCTCCT      1071

GTGCAGGCTC ATGCTGCAGG CGGCCGAGGG CACA                                  1105
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 331 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

```
Met Glu Asn Pro Ser Pro Ala Ala Ala Leu Gly Lys Ala Leu Cys Ala
-23          -20                 -15                 -10

Leu Leu Leu Ala Thr Leu Gly Ala Ala Gly Gln Pro Leu Gly Gly Glu
         -5                  1                   5

Ser Ile Cys Ser Ala Arg Ala Leu Ala Lys Tyr Ser Ile Thr Phe Thr
 10                  15                  20                  25

Gly Lys Trp Ser Gln Thr Ala Phe Pro Lys Gln Tyr Pro Leu Phe Arg
                30                  35                  40

Pro Pro Ala Gln Trp Ser Ser Leu Leu Gly Ala Ala His Ser Ser Asp
            45                  50                  55

Tyr Ser Met Trp Arg Lys Asn Gln Tyr Val Ser Asn Gly Leu Arg Asp
        60                  65                  70

Phe Ala Glu Arg Gly Glu Ala Trp Ala Leu Met Lys Glu Ile Glu Ala
    75                  80                  85

Ala Gly Glu Ala Leu Gln Ser Val His Ala Val Phe Ser Ala Pro Ala
 90                  95                  100                 105

Val Pro Ser Gly Thr Gly Gln Thr Ser Ala Glu Leu Glu Val Gln Arg
                110                 115                 120

Arg His Ser Leu Val Ser Phe Val Val Arg Ile Val Pro Ser Pro Asp
            125                 130                 135
```

```
Trp Phe Val Gly Val Asp Ser Leu Asp Leu Cys Asp Gly Asp Arg Trp
        140                 145                 150

Arg Glu Gln Ala Ala Leu Asp Leu Tyr Pro Tyr Asp Ala Gly Thr Asp
    155                 160                 165

Ser Gly Phe Thr Phe Ser Ser Pro Asn Phe Ala Thr Ile Pro Gln Asp
170                 175                 180                 185

Thr Val Thr Glu Ile Thr Ser Ser Ser Pro Ser His Pro Ala Asn Ser
                190                 195                 200

Phe Tyr Tyr Pro Arg Leu Lys Ala Leu Pro Pro Ile Ala Arg Val Thr
            205                 210                 215

Leu Val Arg Leu Arg Gln Ser Pro Arg Ala Phe Ile Pro Pro Ala Pro
        220                 225                 230

Val Leu Pro Ser Arg Asp Asn Glu Ile Val Asp Ser Ala Ser Val Pro
    235                 240                 245

Glu Thr Pro Leu Asp Cys Glu Val Ser Leu Trp Ser Ser Trp Gly Leu
250                 255                 260                 265

Cys Gly Gly His Cys Gly Arg Leu Gly Thr Lys Ser Arg Thr Arg Tyr
                270                 275                 280

Val Arg Val Gln Pro Ala Asn Asn Gly Ser Pro Cys Pro Glu Leu Glu
            285                 290                 295

Glu Glu Ala Glu Cys Val Pro Asp Asn Cys Val
        300                 305
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GCCATACGGG ATCCCAGCCT CTTGGGGGAG AGTCC 35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GGCATACGTC TAGATTAGAC GCAGTTATCA GGGAC 35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GCCATACGGG ATCCGCCATC ATGGAAAACC CCAGCCCGGC C 41

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GGCATACGTC TAGATTAGAC GCAGTTATCA GGGAC 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

```
Pro Thr Gly Thr Gly Cys Val Ile Leu Lys Ala Ser Ile Val Gln Lys
                  5                  10                  15

Arg Ile Ile Tyr Phe Gln Asp Glu Gly Ser Leu Thr Lys Lys Leu Cys
             20                  25                  30

Glu Gln Asp Pro Thr Leu Asp Gly Val Thr Asp Arg Pro Ile Leu Asp
         35                  40                  45

Cys Cys Ala Cys Gly Thr Ala Lys Tyr Arg Leu Thr Phe Tyr Gly Asn
     50                  55                  60

Trp Ser Glu Lys Thr His Pro Lys Asp Tyr Pro Arg Arg Ala Asn His
 65                  70                  75                  80
```

```
Trp Ser Ala Ile Ile Gly Gly Ser His Ser Lys Asn Tyr Val Leu Trp
                85                  90                  95

Glu Tyr Gly Gly Tyr Ala Ser Glu Gly Val Lys Gln Val Ala Glu Leu
            100                 105                 110

Gly Ser Pro Val Lys Met Glu Glu Glu Ile Arg Gln Gln Ser Asp Glu
        115                 120                 125

Val Leu Thr Val Ile Lys Ala Lys Ala Gln Trp Pro Ser Trp Gln Pro
    130                 135                 140

Val Asn Val Arg Ala Ala Pro Ser Ala Glu Phe Ser Val Asp Arg Thr
145                 150                 155                 160

Arg His Leu Met Ser Phe Leu Thr Met Met Gly Pro Ser Pro Asp Trp
                165                 170                 175

Asn Val Gly Leu Ser Ala Glu Asp Leu Cys Thr Lys Glu Cys Gly Trp
            180                 185                 190

Val Gln Lys Val Val Gln Asp Leu Ile Pro Trp Asp Ala Gly Thr Asp
        195                 200                 205

Ser Gly Val Thr Tyr Glu Ser Pro Asn Lys Pro Thr Ile Pro Gln Glu
    210                 215                 220

Lys Ile Arg Pro Leu Thr Ser Leu Asp His Pro Gln Ser Pro Phe Tyr
225                 230                 235                 240

Asp Pro Glu Gly Gly Ser Ile Thr Gln Val Ala Arg Val Val Ile Glu
                245                 250                 255

Arg Ile Ala Arg Lys Gly Glu Gln Cys Asn Ile Val Pro Asp Asn Val
            260                 265                 270

Asp Asp Ile Val Ala Asp Leu Ala Pro Glu Glu Lys Asp Glu Asp Asp
        275                 280                 285

Thr Pro Glu Thr Cys Ile Tyr Ser Asn Trp Ser Pro Trp Ser Ala Cys
    290                 295                 300

Ser Ser Ser Thr Cys Glu Lys Gly Lys Arg Met Arg Gln Arg Met Leu
305                 310                 315                 320

Lys Ala Gln Leu Asp Leu Ser Val Pro Cys Pro Asp Thr Gln Asp Phe
                325                 330                 335

Gln Pro Cys Met Gly Pro Gly Cys Ser Asp Glu Asp Gly Ser Thr Cys
            340                 345                 350

Thr Met Ser Glu Trp Ile Thr Trp Ser Pro Cys Ser Val Ser Cys Gly
        355                 360                 365

Met Gly Met Arg Ser Arg Glu Arg Tyr Val Lys Gln Phe Pro Glu Asp
    370                 375                 380

Gly Ser Val Cys Met Leu Pro Thr
385                 390
```

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide having at least a 70% identity to a polynucleotide encoding a polypeptide comprising amino acids 1 to 308 set forth in SEQ ID NO:2;

(b) a polynucleotide which is complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 15 bases of the polynucleotide of (a) or (b).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of Claim 2 encoding a polypeptide comprising amino acids 1 to 308 of SEQ ID NO:2.

5. The polynucleotide of claim 2 comprising nucleotide 1 to nucleotide 1105 set forth in SEQ ID NO:1.

6. The polynucleotide of Claim 2 comprising nucleotide 97 to 1011 set forth in SEQ ID NO:1.

7. An isolated polynucleotide comprising a member selected from the group consisting of:

(a) a polynucleotide having at least a 70% identity to a polynucleotide encoding the same mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97343;

(b) a polynucleotide complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 15 bases of the polynucleotide of (a) or (b).

8. A vector comprising the DNA of Claim 2.

9. A host cell comprising the vector of Claim 8.

10. A process for producing a polypeptide comprising: expressing from the host cell of Claim 9 a polypeptide encoded by said DNA.

11. A process for producing a cell which expresses a polypeptide comprising genetically engineering the cell with the vector of Claim 8.

12. A polypeptide comprising a member selected from the group consisting of:

(a) a polypeptide having an amino acid sequence set forth in SEQ ID NO:2;

(b) a polypeptide comprising amino acids 1 to 308 set forth in SEQ ID NO:2; and

(c) a polypeptide which is at least 70% identical to the polypeptide of (a) or (b).

13. The polypeptide of Claim 12 wherein the polypeptide comprises an amino acid sequence set forth in SEQ ID NO:2.

14. The polypeptide of Claim 12 wherein the polypeptide comprises amino acids 1 to amino acid 308 of SEQ ID NO:2.

15. An antagonist against the polypeptide of claim 12.

16. An antibody against the polypeptide of claim 12.

17. A method for the treatment of a patient having need of NAF-1 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 12.

18. The method of Claim 17 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

19. A method for the treatment of a patient having need to inhibit NAF-1 polypeptide comprising: administering to the patient a therapeutically effective amount of the compound of Claim 15.

20. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 12 comprising:

determining a mutation in a nucleic acid sequence encoding said polypeptide.

21. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 12 in a sample derived from a host.

22. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 12 comprising: contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable NAF-1 polypeptide and a compound under conditions to permit binding to the receptor; and

determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the NAF-1 with the receptor.

FIG. 1A

```
1    CGCTGCTCCTGCCGGGTGATGGAAAACCCCAGCCCGGCCGCCGCCCTGGGCAAGGCCCTC   60
1                    M  E  N  P  S  P  A  A  A  A  L  G  K  A  L    20

61   TGCGCTCTCCTCCTGGCCACTCTCGGCGCCGCCGGCCAGCCTCTTGGGGGAGAGTCCATC  120
21   C  A  L  L  L  A  T  L  G  A  A  G  Q  P  L  G  G  E  S  I    40

121  TGTTCCGCCAGAGCCCTGGCCAAATACAGCATCACCTTCACGGGCAAGTGGAGCCAGACG  180
41   C  S  A  R  A  L  A  K  Y  S  I  T  F  T  G  K  W  S  Q  T    60

181  GCCTTCCCCAAGCAGTACCCCCTGTTCCGCCCCCCTGCGCAGTGGTCTTCGCTGCTGGGG  240
61   A  F  P  K  Q  Y  P  L  F  R  P  P  A  Q  W  S  S  L  L  G    80

241  GCCGCGCATAGCTCCGACTACAGCATGTGGAGGAAGAACCAGTACGTCAGTAACGGGCTG  300
81   A  A  H  S  S  D  Y  S  M  W  R  K  N  Q  Y  V  S  N  G  L   100

301  CGCGACTTTGCGGAGCGCGGCGAGGCCTGGGCGCTGATGAAGGAGATCGAGGCGGCGGGG  360
101  R  D  F  A  E  R  G  E  A  W  A  L  M  K  E  I  E  A  A  G   120

361  GAGGCGCTGCAGAGCGTGCACGCGGTGTTTTCGGCGCCCGCCGTCCCCAGCGGCACCGGG  420
121  E  A  L  Q  S  V  H  A  V  F  S  A  P  A  V  P  S  G  T  G   140

421  CAGACGTCGGCGGAGCTGGAGGTGCAGCGCAGGCACTCGCTGGTCTCGTTTGTGGTGCGC  480
141  Q  T  S  A  E  L  E  V  Q  R  R  H  S  L  V  S  F  V  V  R   160

481  ATCGTGCCCAGCCCCGACTGGTTCGTGGGCGTGGACAGCCTGGACCTGTGCGACGGGGAC  540
161  I  V  P  S  P  D  W  F  V  G  V  D  S  L  D  L  C  D  G  D   180
```

MATCH WITH FIG. 1B

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MATCH WITH FIG. 1A

FIG. 1B

```
541  CGTTGGCGGGAACAGGCGGCGCTGGACCTGTACCCCTACGACGCCGGGACGGACAGCGGC  600
181  R  W  R  E  Q  A  A  L  D  L  Y  P  Y  D  A  G  T  D  S  G   200

601  TTCACCTTCTCCTCCCCCAACTTCGCCACCATCCCGCAGGACACGGTGACCGAGATAACG  660
201  F  T  F  S  S  P  N  F  A  T  I  P  Q  D  T  V  T  E  I  T   220

661  TCCTCCTCTCCCAGCCACCCGGCCAACTCCTTCTACTACCCGCGGCTGAAGGCCCTGCCT  720
221  S  S  S  P  S  H  P  A  N  S  F  Y  Y  P  R  L  K  A  L  P   240

721  CCCATCGCCAGGGTGACACTGGTGCGGCTGCGACAGAGCCCCAGGGCCTTCATCCCTCCC  780
241  P  I  A  R  V  T  L  V  R  L  R  Q  S  P  R  A  F  I  P  P   260

781  GCCCCAGTCCTGCCCAGCAGGGACAATGAGATTGTAGACAGCGCCTCAGTTCCAGAAACG  840
261  A  P  V  L  P  S  R  D  N  E  I  V  D  S  A  S  V  P  E  T   280

841  CCGCTGGACTGCGAGGTCTCCCTGTGGTCGTCCTGGGGACTGTGCGGAGGCCACTGTGGG  900
281  P  L  D  C  E  V  S  L  W  S  S  W  G  L  C  G  G  H  C  G   300

901  AGGCTCGGGACCAAGAGCAGGACTCGCTACGTCCGGGTCCAGCCCGCCAACAACGGGAGC  960
301  R  L  G  T  K  S  R  T  R  Y  V  R  V  Q  P  A  N  N  G  S   320

961  CCCTGCCCCCGAGCTCGAAGAAGAGGCTGAGTGCGTCCCTGATAACTGCGTCTAAGACCAG 1020
321  P  C  P  E  L  E  E  E  A  E  C  V  P  D  N  C  V  *         340

1021 AGCCCCGCAGCCCCTGGGGCCCCCCGGAGCCATGGGGTGTCGGGGGCTCCTGTGCAGGCT 1080
1081 CATGCTGCAGGCGGCCGAGGGCACA                                    1105
```

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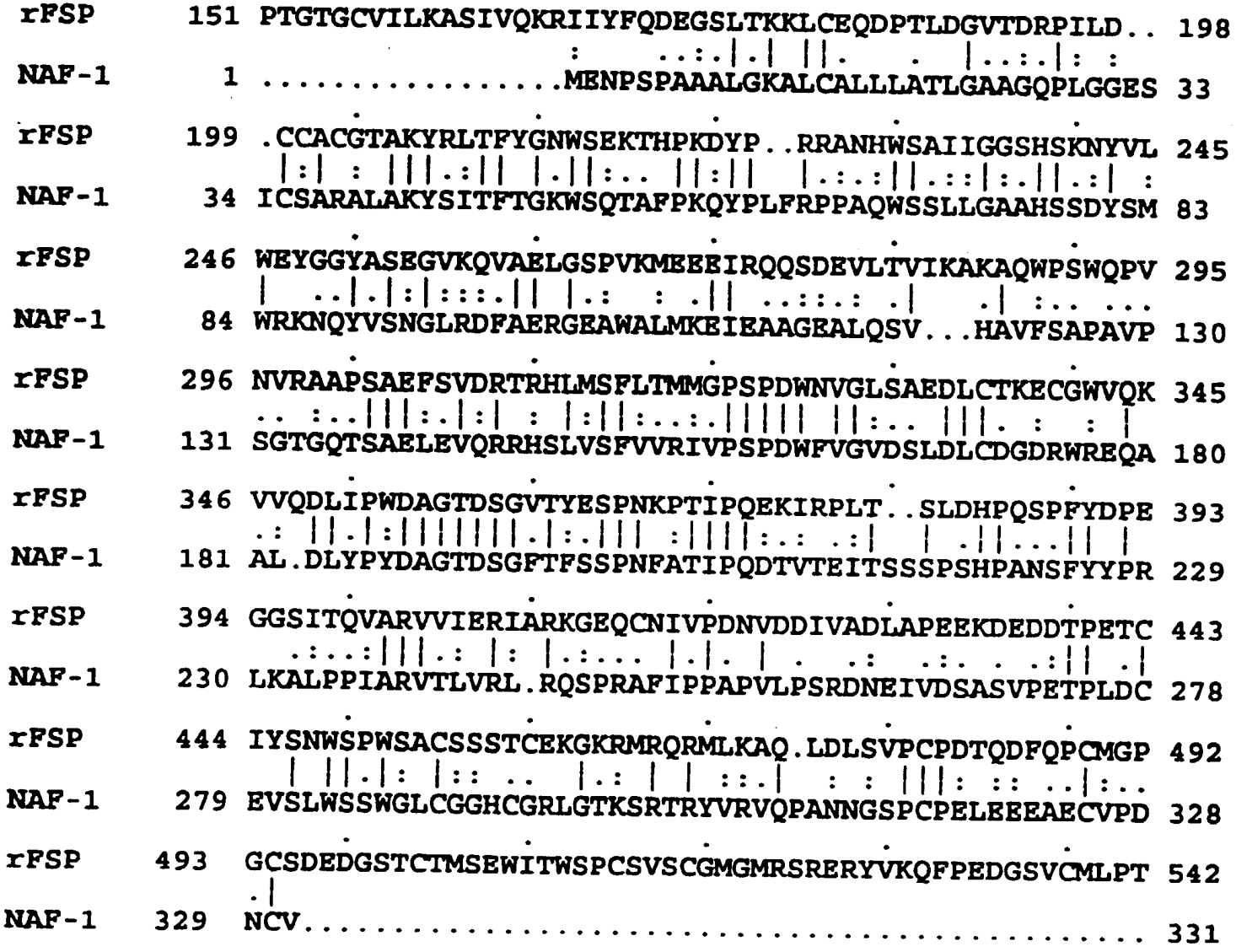
FIG. 2



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01857

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/10, 15/12; C07K 19/00

US CL :435/69.1, 240.2, 252.3, 320.1; 530/350, 399; 536/23.1, 23.5, 24.31

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, 240.2, 252.3, 320.1; 530/350, 399; 536/23.1, 23.5, 24.31

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.
X	US, A, 5,279,966 (JESSELL ET AL.) 19 January 1994, column 59 lines 59-67, column 60, lines 50-67, column 61, lines 1-12 and column 62, lines 1-12.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search

13 JUNE 1996

Date of mailing of the international search report

28 JUN 1996

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/01857

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

APS, DIALOG, SCISEARCH, BIOSIS, MEDLINE, JPO, STN
search terms: neuronal attractant factor, F-spondin, neural growth factors, axonal growth factors, floor plate specific genes, chemoattractants, chemotaxis(tic), neuronal tube development, fasciculate, epithelioid sarcoma, Klar, TRKA, Danforth's short tail mice

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-14, drawn to an isolated polynucleotide, a vector, a host cell, a process for using the host cell, a process for using cells and a polypeptide.

II. Claims 15 and 19, drawn to an antagonist and a method of treatment comprising administering an antagonist.

III. Claim 16, drawn to an antibody against a polypeptide.

IV. Claim 17, drawn to a method for the treatment of a patient comprising administering a polypeptide.

V. Claim 18, drawn to a method for the treatment of a patient comprising administering DNA.

VI. Claim 20, drawn to a process for diagnosing a disease comprising determining a mutation.

VII. Claim 21, drawn to a diagnostic process comprising analyzing for a polypeptide.

VIII. Claim 22, drawn to a method for identifying antagonists.

The DNA and polypeptide compositions of Group I have materially different chemical structures and biological functions from the antagonist of Group II and antibody of Group III. The special technical features by which the DNA and polypeptide of Group I are defined distinguish them from the special technical features which define the antagonist of Group II and the antibody of Group III. The method of each group, I and IV-VIII, is materially different from the method in any other group because each is practiced with materially different process steps; the process steps are the special technical features which distinguish each method from the others. Because the process steps do not share the same or a corresponding special technical feature, unity of the invention is lacking.