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(54) Title: EXPRESSION OF NEUROTROPHIC FACTORS WITH HETEROLOGOUS PREPRO REGIONS

(57) Abstract

The present invention relates to chimeric prepro proteins or prepro peptides comprising neurotrophic factors, and the use of such precursors and their nucleic acid sequences to produce proteins or peptides which have one or more biological activities of a neurotrophin. The chimeric prepro molecules provided by the present invention contain a heterologous prepro region fused to a mature neurotrophin sequence or biologically active portion or derivative thereof. The mature neurotrophin sequences that can be used according to the present invention are those of the NGF/BDNF family of homologous molecules including but not limited to NGF, BDNF, NT-3, and NT-4. Similarly, the prepro regions can be derived from those neurotrophin molecules of the NGF/BDNF family including but not limited to NGF, BDNF, NT-3, and NT-4.

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EXPRESSION OF NEUROTROPHIC FACTORS WITH HETEROLOGOUS PREPRO REGIONS

1. INTRODUCTION

The present invention relates to the construction and expression in eukaryotic host cells of novel chimeric prepro proteins or prepro peptides expressing bioactive neurotrophic factors. The invention is based, in substantial part, on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of a first neurotrophic factor fused to the mature protein, or portion thereof, of a second, different neurotrophic factor undergo efficient post-translational processing resulting in an increased level of expression of the bioactive second neurotrophic factor protein.

2. BACKGROUND OF THE INVENTION

2. 1. NEUROTROPHIC FACTORS

The development and maintenance of the nervous system depends on proteins known as neurotrophic factors. A neurotrophic factor is a cytokine, a protein which acts as a messenger and communicates with other cells in the ongoing coordination and regulation of biological functions. Neurotrophic factors promote the survival and/or differentiation of components of the nervous system. Widespread neuronal cell death accompanies normal development of the central and peripheral nervous systems, and apparently plays a crucial role in regulating the number of neurons which project to a given target field (Berg, D. K., 1982, Neuronal Development 297-331). Ablation and transplantation studies of peripheral target tissues during development have shown that neuronal cell death

results from the competition among neurons for limiting amounts of survival factors ("neurotrophic factors") produced in their projection fields. Important neurotrophic factors identified to date include nerve growth factor (NGF; Levi-Montalcini and Angeletti, 1968, Phys. Rev. 48:534); neurotrophin-3 (NT-3; Hohn et al., 1990, Nature 344:339; Maisonpierre et al., 1990, Science 247:1446), brain-derived neurotrophic factor (BDNF; Barde et al., 1982, EMBO J. 1:549), neurotrophin-4 (NT-4; Hallbook et al., 1991, Neuron 6:845-858), and ciliary neurotrophic factor (CNTF; Lin et al., 1979, Science 246:1023).

Neurotrophins are generally synthesized in vivo as "prepro" precursor proteins. The "prepro" region refers to the NH_2-terminus of the precursor which is proteolytically removed during biosynthesis of the mature, biologically active form of the protein. The "pre" region refers to the signal sequence normally removed by proteolytic processing during translocation across the cell membrane to yield a "pro"-protein; the "pro" region is then removed by proteolytic processing to yield the mature form (see e.g., Darnell et al., 1990, Molecular Cell Biology 2d ed., Scientific American Books, pp. 650-657).

2. 1. 1. NERVE GROWTH FACTOR

Nerve growth factor (NGF) is by far the most fully characterized of these neurotrophic molecules and has been shown, both in vitro and in vivo, to be essential for the survival of sympathetic and neural crest-derived sensory neurons during early development of both chick and rat (Levi-Montalcini and Angeletti, 1963, Develop. Biol. 7:653-659; Levi-Montalcini et al., 1968, Physiol. Rev. 48:524-569). Until recently, almost all studies of NGF had focused on its role in

the peripheral nervous system, but it now appears that NGF also influences the development and maintenance of specific populations of neurons in the central nervous system (Thoenen et al., 1987, Rev. Physiol. Biochem. Pharmacol. 109:145-178; Whittemore and Seiger, 1987, Brain Res. Rev. 12:439-464).

The abundance of NGF protein in mouse submaxillary gland allowed the primary amino acid sequence to be determined by relatively conventional protein chemistry (Angeletti and Bradshaw, 1971, Proc. Natl. Acad. Sci. 68:2417-2420). The NGF gene has now been cloned from many species, including mouse (Scott et al., 1983, Nature 302:538-540, human (Uilrich et al., 1983, Nature 303: 821-825), cow and chick (Meier et al., 1986, EMBO J. 5:1489-1493), and rat (Whittemore et al., 1988, J. Neurosci. Res., 20:402-410) using conventional molecular biology techniques based on the availability of the protein sequence of mouse NGF to design suitable oligonucleotide probes.

The mouse NGF gene encompasses approximately 45 kb, containing several small 5' exons, with alternating splicing resulting in four distinct mRNA species (Serby, et al., 1987, Mol. Cell. Biol. 7:3057-3064). Two major transcripts result in a "long" and "short" NGF prepropeptide (Edwards, et al., 1986, Nature 319:784-787; Serby, et al., 1987, Mol. Cell. Biol. 7:3057-3064). The "short" precursor contains a conventional signal sequence (pre-region) at the NH_2-terminus which flanks the pro-region. The "long" precursor contains an additional "pro-region" at its NH_2-terminus (see e.g., Suter et al, 1991, EMBO J. 10:2395-2400, Figure 1). To date, no functional distinction between the "long" and "short" NGF prepro precursor has been elucidated. However, the shorter mRNA transcript is more abundant in most

tissue (Edwards et al., 1986, J. Biol. Chem. 263:6810-6815).

The biologically active form of mouse NGF is a 7S complex, comprising a dimer of a fully processed mature form of β-NGF along with two members of the kallikrein family of serine proteases, the α-subunit and γ-subunit of NGF (Varon et al., Biochemistry 7:1296-1303; Mason et al., 1983, Nature 303:300-307). The translation, processing and secretion of the NGF precursor to form a biologically active form of NGF is well documented. Darling, et al. (1983, Cold Spring Harbor Symp. Quan. Biol. 48:427-433), on the strength of the reported cDNA sequence encoding mouse NGF (Scott, et al., 1983, Nature 302: 538-540), utilized an in vitro cell free translation system to identify key intermediates in the biosynthesis of the 7S complex of NGF. The signal sequence of the prepro NGF precursor is removed via proteolytic processing to yield a pro-NGF species of approximately 31 kD. The pro-region of the pro-NGF intermediate contains a pair of arginine residues known to be endoproteolytic processing sites. Proteolytic processing at either of these residues results in an additional major (21 kD) and minor (18.5 kD) intermediate species. The mature form of NGF can be proteolytically derived from either of the above-mentioned intermediate species. At some point in the biosynthesis of the mature form of NGF, a COOH-terminal dipeptide (arg-gly) is proteolytically released.

The γ-subunit has been shown in vivo to proteolytically cleave the pro-NGF precursor to the mature form of NGF (Edwards, et al., 1988, J. Biol. Chem. 263: 6810-6815). Attempts to mimic the process in vitro were unsuccessful, resulting in unfaithful processing of the pro-NGF precursor, presumably due to

aberrant folding of the in vitro translation product. Silen and Agard (1989, Nature 341:462-464) demonstrated that the pro region may facilitate proper folding of the α-lytic protease precursor. Therefore, the pro region of the NGF precursor may also be required for proper folding prior to endoproteolytic processing to the mature form and association into the biologically active 7S NGF complex. Support for this hypothesis is documented in Suter et al. (1991, EMBO J. 10:2395-2400), who assigned functions for two partially conserved domains within the pro-region of NGF. Domain I was shown to be essential for NGF expression in COS cells. Additionally, Domain II, located in the NGF pro-region proximal to the mature coding region, was found to be involved in proteolytic processing.

Endoproteolytic processing of pro-NGF in vivo has recently been shown to be controlled by the human fur gene product, a membrane associated endoprotease sharing structural homology with the KEX2 gene, which encodes a yeast endoprotease (Bresnahan, et al., 1990, J. Cell Biol. 111:2851-2859).

Therefore, initiation of biosynthesis of the active form of mouse NGF involves the transcription of the NGF gene and possible alternative splicing of the transcription product to generate mRNA's capable of translation of either a long or short NGF preproprecursor. The long or short prepro NGF precursor is subsequently subjected to a series of endoproteolytic processing events, possibly induced by proper folding of the precursor via the structural characteristics of the pro-region, resulting in the mature form of NGF.

2. 1. 2. BRAIN-DERIVED NEUROTROPHIC FACTOR

Using pig brain as a starting material, Barde et al. (1982, EMBO J. 1:549-553) reported a factor, now termed brain-derived neurotrophic factor (BDNF), which appeared to promote the survival of dorsal root ganglion neurons from E10/E11 chick embryos. The neurotrophic activity was found to reside in a highly basic protein (isoelectric point, pI 10.1) which migrated during sodium dodecyl sulfate (SDS) gel electrophoresis as a single band of 12.3 kD. It was noted that the highly basic nature and molecular size of BDNF were very similar to the NGF monomer.

The cloning of the BDNF gene was first performed as described in copending U.S. Patent Application Serial Number 07/400,591, filed August 30, 1989, which is incorporated by reference in its entirety herein (see also PCT International Publication No. WO 91/03568, published March 21, 1991). Complete cDNA and/or genomic BDNF genes were cloned from a variety of species, including human, pig, rat, and mouse and the sequences of these genes were determined. Expression of recombinant BDNF was achieved in COS cells.

The first demonstration of neuronal specificity of BDNF distinct from that of NGF was the demonstration in vitro that purified BDNF supports the survival of 40-50% of sensory neurons dissociated from the neural placode-derived nodose ganglion of the chick embryo at E6, E9 or E12 (Lindsay et al., 1985, J. Cell. Sci. Supp. 3:115-129). NGF was without apparent effect on these neurons either by itself or in conjunction with BDNF. It was later shown in explant culture studies that BDNF appeared to support survival and neurite outgrowth from other neural

placode-derived sensory ganglia, including the petrosal, geniculate and ventrolateral trigeminal ganglia (Davies et al., 1986, J. Neurosci. 6:1897-1904), none of which have been found to be sensitive to NGF. In addition to its effects on cultured neurons from peripheral ganglia, BDNF was found to stimulate survival and neuronal differentiation of cells cultured from quail neural crest (Kalcheim and Gendreau, 1988, Develop. Brain Res. 41:79-86).

Two recent studies with BDNF (Kalcheim, et al., 1987, EMBO J. 6:2871-2873; Hofer and Barde, 1988, Nature 331:261-262) have, however, indicated a physiological role of BDNF in avian PNS development. In addition to its effect on peripheral sensory neurons of both neural crest and neural placode origin, BDNF has been found to support the survival of developing CNS neurons; Johnson et al. (1986, J. Neurosci. 6:3031-3938) presented data indicating that BDNF supports the survival of retinal ganglion cells cultured from E17 rat embryos.

In addition to its effects on the survival of developing neurons in culture, BDNF has been shown to have effects on cultured adult peripheral and central nervous system neurons.

Analysis of the predicted primary structure of mature BDNF has revealed a striking similarity to NGF; with only three gaps introduced into the NGF sequences to optimize matching, 51 identities are common to the various NGFs (from snake to man) and BDNF. Importantly, these identities include six cysteine residues.

2. 1. 3. NEUROTROPHIN-3

Another member of the neurotrophin family, termed neurotrophin-3, was discovered, and the NT-3

gene was cloned from mouse, rat, and human (see U.S. Patent Application Serial No. 07/490,004, filed March 7, 1990, incorporated by reference in its entirety herein; see also PCT International Publication No. WO 91/03569, published March 21, 1991). The overall structure of mature mouse NT-3 protein, consisting of 119 amino acids with a computed pI of about 9.5, was found to resemble that established for NGF and BDNF; a putative signal sequence of 18 amino acids (showing 5 and 9 amino acid identities with BDNF and NGF, respectively) appears to be followed by a prosequence of 121 amino acids (as compared with a prosequence of 103 amino acids in mouse NGF and a prosequence of 112 amino acids in mouse BDNF). A comparison between mature mouse NGF, BDNF, and NT-3 revealed 54 amino acid identities. All 6 cysteine residues, known in NGF and BDNF to be involved in the formation of disulfide bridges (Leibrock et al., 1989, Nature 341:149-152; Angeletti, 1973, Biochem. 12:100-115), are amongst the conserved residues. Similarly, mature rat NT-3 appears to share 57% amino acid homology with rat NGF, and 58% amino acid homology with rat BDNF; 57 of the 120 residues (48%) appear to be shared by all three proteins. Again, the six cysteine residues of rat NGF and BDNF were found to be absolutely conserved in rat NT-3, and regions of greatest homology between the three proteins appear to cluster around these cysteine residues.

In addition to the homology between NT-3, NGF, and BDNF within a species, a high degree of conservation in nucleic acid sequence was observed between rat and human NT-3 within the region encoding the mature polypeptide (119 amino acids). The deduced amino acid sequences of mature rat and human (as well

as mouse NT-3) appear absolutely identical, reminiscent of the high degree of conservation of BDNF, which shows complete identity in the amino acid sequence of the mature polypeptide among rat, mouse, human, and pig. By contrast, the amino acid sequences of mature human NGF and rodent NGF (mouse or rat) differ by approximately 10 percent.

Studies of the neurotrophic activity of NT-3 have indicated that NT-3 is capable of promoting survival and neurite outgrowth of dissociated dorsal root ganglion neurons in culture. Furthermore, NT-3 was observed to promote neurite outgrowth from both nodose ganglion and sympathetic ganglion explants, whereas BDNF promoted outgrowth from nodose ganglion but not sympathetic ganglion, and NGF promoted outgrowth from sympathetic ganglion but not nodose ganglion explants. Therefore, NT-3 appears to have a broader specificity of action than either BDNF or NGF.

2.1.4 NEUROTROPHIN-4

Neurotrophin-4 is a novel member of the NGF family that has recently been cloned and isolated (Hallbook et al., 1991, Neuron 6:845-858). PCR fragments corresponding to the NT-4 gene from Xenopus and viper were obtained, and a genomic Xenopus clone was subsequently isolated. Nucleotide sequence analysis of this clone revealed an open reading frame for a protein of 236 amino acids, with several structural features similar to those of NGF, BDNF and NT-3. These features include a putative amino-terminal signal sequence and a potential N-glycosylation site near a proteolytic cleavage site. As is true for NGF, BDNF, and NT-3, the entire Xenopus pre-pro-NT-4 protein is encoded in one single exon.

2.2 PRODUCTION OF NEUROTROPHINS

Various expression vectors and hosts have been utilized in attempts to produce recombinant neurotrophins.

All using animal cell expression systems (mammalian kidney cells), Liebrock et al. [Nature 341:149 (1989)] reported the expression of biologically active pig BDNF, and Rosenthal et al. [Neuron 4: 767 (1990)], Maisonpierre et al. [Science 247:1446 (1990)] and Hohn et al. [Nature 344:339 (1990)] separately reported the expression of biologically active NT-3 of various species. In addition, Chan et al. [EP Publication No. 370171, published May 1990] reported the expression of biologically active mature human BDNF from insect cells by way of a baculovirus expression system.

Regarding microbial production of neurotrophins, Iwai et al. [Chem. Pharm. Bull. 34:4727 (1986)] reported the expression of synthetic "genes" for human NGF and a fusion thereof in E. coli. The product was only characterized by molecular weight, after treatment with a reducing agent, and there was no information regarding the presence of biological activity.

Dicou et al. [J. Neuroscience Res. 22:13 (1989)] reported the separate expression of mouse and hNGF fusions in E. coli. Dicou et al. (1989, J. Neurosci. Res. 22:13-19) fused the complete mouse prepro-nerve growth factor DNA sequence to the carboxyl terminus of the beta-galactosidase gene of Escherichia coli, and also fused a genomic DNA fragment corresponding to codons 11 to 106 of the human nerve growth factor gene to the fifth codon of the amino terminus of beta-galactosidase. Both bacterial vectors were associated with the expression

of large amounts of the chimeric proteins. Although after bacterial cell lysis most of the chimeric mouse prepro-nerve growth factor appeared to be insoluble, the majority of human chimeric beta-nerve growth factor seemed to exist in the supernatant. Neurotrophic activity was not reported.

Finally, Hu et al. [Gene 70:57 (1988); and Abstract 343.16 of the 20th Ann. Meeting of the Soc. for Neuroscience (1990)] reported expression of mouse NGF in E. coli.

3. SUMMARY OF THE INVENTION

The present invention relates to novel chimeric prepro proteins or prepro peptides comprising bioactive neurotrophic factors, and the use of such precursors and their nucleic acid sequences to produce proteins or peptides which have one or more biological activities of a neurotrophin. The chimeric prepro molecules provided by the present invention contain a heterologous prepro region fused to a mature neurotrophin sequence or biologically active portion or derivative thereof. The mature neurotrophin sequences that can be used according to the present invention are those of the NGF/BDNF family of homologous molecules including but not limited to NGF, BDNF, NT-3 and NT-4. Similarly, the prepro regions can be derived from those neurotrophin molecules of the NGF/BDNF family including but not limited to NGF, BDNF, NT-3 and NT-4. The invention is based, in substantial part, on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of nerve growth factor fused to the mature portion of brain-derived neurotrophic factor (prepro NGF/BDNF) are more efficiently processed by a eukaryotic host cell than homologous prepro brain-

derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cells expressing chimeric prepro NGF/BDNF secrete only the mature form of BDNF into the media. According to the present invention, the "long" or "short" prepro regions of NGF can be utilized in the construction of chimeric neurotrophic genes.

The invention is also based on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of NT-3 fused to the mature coding region of brain-derived neurotrophic factor (prepro NT-3/BDNF) are more efficiently processed than homologous prepro brain-derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cell expressing chimeric NT-3/BDNF secrete only the mature form of BDNF into the media.

The present invention provides for nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides, and for methods of expressing these chimeric neurotrophic proteins and peptides by use of such nucleic acids.

4. DESCRIPTION OF THE FIGURES

Figure 1. Polyacrylamide gel electrophoresis of recombinant BDNF, NGF, and chimeric precursor forms. Cell supernatants from metabolically labeled CHO-DG44 cells stably transfected with various constructs were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Lane 1: wild-type control CHO-DG44 cells. The following constructs were used: expression vector pCDM8 containing the human NGF gene (lane 2); short

prepro BDNF construct (lane 3); long prepro NGF/BDNF chimeric construct (lane 4). Lane 5: molecular weight markers.

Figure 2. Bioactivity of recombinant BDNF. Crude supernatants from transfected CHO cell lines were assayed with embryonic (E8) chick dorsal root ganglia and neurite outgrowth was scored. Closed diamonds: cell line DGC-N/B-2.5-#23 (containing long prepro NGF/BDNF chimeric construct). Dotted squares: cell line DGZ1000-B-3-2.5 (containing short prepro BDNF construct).

Figure 3. Sequence of human BDNF cDNA (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2), and comparison of DNA sequences from pig (SEQ ID NO:3 and SEQ ID NO:4), rat (SEQ ID NO:5 AND SEQ ID NO:6), and chicken (SEQ ID NO:7 and SEQ ID NO:8). The figure shown is from PCT International Publication No. WO 91/03568, published March 21, 1991.

Figure 4. Nucleotide (SEQ ID NO:9) and deduced amino acid (SEQ ID NO:10) sequence of human NGF. -187 through -1 indicates the long prepro region. The sequence information is from EP Publication 121,338, published October 10, 1984, by Gray and Ullrich.

Figure 5. Aligned DNA sequences of the rat (SEQ ID NO:11) and human (SEQ ID NO:13) NT-3 genes. The predicted translation start site is indicated by "PREPRO--" and the predicted start of the mature NT-3 is indicated by "MATURE--". The mature rat (SEQ ID NO:12) and human (SEQ ID NO:14) NT-3 proteins have identical amino acid sequences whereas their prepro regions differ at 11 positions, which are underlined. The figure shown is from PCT International Publication No. WO 91/03569, published March 21, 1991.

Figure 6. DNA fragment 3 (SEQ ID NO:15 and 16), utilized in the NT-3/BDNF chimeric construction, corresponding to 35 amino acids of the NT-3 prepro region (SEQ ID NO:17).

Figure 7. Western blot analysis of conditioned media from CHO cell clones expressing either the original prepro BDNF (lane 2-11) or the chimeric prepro NT-3/BDNF (lane 12-19). Lane 1 was loaded with 450 ng of purified mature human BDNF. Lane 20 was loaded with prestained low molecular weight markers from BRL. Lane 8 and lane 16 represent non-producing clones from each transfection.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel chimeric prepro proteins or prepro peptides comprising bioactive neurotrophic factors, and the use of such precursors and their nucleic acid sequences to produce proteins or peptides which have one or more biological activities of a neurotrophin. The chimeric prepro molecules provided by the present invention contain a heterologous prepro region fused to a mature neurotrophin sequence or biologically active portion or derivative thereof. The mature neurotrophin sequences which can be used according to the present invention are those of the NGF/BDNF family of homologous molecules including but not limited to NGF, BDNF, NT-3 and NT-4. Similarly, the prepro regions can be derived from those neurotrophin molecules of the NGF/BDNF family including but not limited to NGF, BDNF, NT-3 and NT-4. The invention is based, in substantial part, on the discovery that chimeric prepro proteins or prepro peptides comprising the prepro region of nerve growth factor and the mature portion of brain-derived neurotrophic factor (prepro

NGF/BDNF) or the prepro region of NT-3 and the mature portion of brain-derived neurotrophic factor (prepro NT-3/BDNF) are more efficiently processed by a eukaryotic host cell than homologous prepro brain-derived neurotrophic factor (prepro BDNF). It is further based on the discovery that stably transfected and amplified eukaryotic host cells expressing chimeric prepro NGF/BDNF or NT-3/BDNF secrete only the mature form of BDNF into the media. The post-translational processing of homologous prepro BDNF is highly inefficient. In contrast, a member of the same neurotrophin gene family, NGF, is efficiently processed. Only the mature bioactive form of NGF is secreted into the host cell media following transient transfection. The BDNF processing problem has carried through in the generation of stable host cell lines for the production of mature bioactive BDNF. The present invention provides a novel solution to this processing problem by expression of chimeric constructs, which in a specific embodiment contains the long prepro region of NGF fused in frame to mature BDNF and in another specific embodiment contains the prepro region of NT-3 fused in frame to mature BDNF.

The present invention provides for nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides, and for methods of expressing these chimeric neurotrophic proteins and peptides by use of such nucleic acids.

Expression of nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides according to the present invention provide significant advantages relative to the use of nucleic acids encoding homologous neurotrophic prepro proteins or prepro peptides. Production of chimeric neurotrophic prepro proteins or prepro peptides

provides for increased expression levels of the bioactive neurotrophic factor. This increased level of expression should additionally provide for better bioactive neurotrophic factor purification schemes in that contaminating unprocessed forms of the expressed neurotrophic factors are not apparent in the crude supernatants.

5.1 THE EXPRESSION PRODUCTS OF THE PRESENT INVENTION

The bioactive proteins which can be obtained according to the present invention are the mature neurotrophic factors which are members of the neurotrophin gene family, or biologically active portions or derivatives thereof. The term "biologically active" as used herein refers to the ability to express one or more biological activities of the full-length mature neurotrophin. Such neurotrophins include but are not limited to mature BDNF, NT-3, NGF and NT-4 and such other members as are identified by those methods utilized to determine members of the neurotrophin gene family (e.g., using molecular probes, generated by PCR, corresponding to regions of homology within the family; see PCT Publication WO 91/03569).

The DNA coding sequences for various neurotrophin proteins, which can be expressed using the present invention, are available. See, Ullrich et al. (Nature 303:821 (1983); E.P. Publication 121,338, published October 10, 1984) regarding hNGF coding sequences and, e.g., Meier et al. (EMBO J. 5:1489 (1986)) and Schwarz et al. (J. Neurochem. 52:1203 (1989)) regarding NGF cDNAs from various other species; ATCC plasmid strain phBDNF-C-1 (Accession No. 4068) regarding a hBDNF cDNA clone and, e.g., Leibrock et al., infra, regarding a pig BDNF cDNA; and ATCC

plasmid strain pC8-hN3 (P1) (Accession No. 40765) regarding a human NT-3 cDNA clone and Maisonpierre et al. (Science 247:1446 (1990)) and Hohn et al. (Nature 344:339 (1990)) regarding NT-3 coding sequences from various other species. The cloning of the human (Rosenthal et al., Neuron 4:767 (1990)) as well as rat (Maisonpierre et al., infra) NT-3 genes has been reported. Furthermore, the nucleotide and amino acid sequences for BDNF are disclosed in PCT Publication WO 91/03568, published March 21, 1991 and copending U.S. application Serial No. 570,657 filed August 20, 1990; the nucleotide and amino acid sequences for NT-3 are disclosed in PCT Publication WO 91/03569 published March 21, 1991 and copending application Serial No. 570,189, filed August 20, 1990). In addition, nucleotide and amino acid sequences for BDNF (SEQ ID NO:1-8), NGF (SEQ ID NO:9-10), and NT-3 (SEQ ID NO:11-14) are presented in Figures 3, 4, and 5, respectively, herein.

In addition, a neurotrophin gene from any organism may be identified using the regions of homology shared by any two members of the BDNF/NGF/NT-3/NT-4 family of molecules using the methods set forth above. For example, and not by way of limitation, a novel neurotrophin may be identified and cloned by BDNF/NGF/NT-3/NT-4 synthesizing degenerate oligonucleotides corresponding to segments of protein sequences highly conserved between any two neurotrophins. These oligonucleotides can then be used as primers in polymerase chain reaction (PCR) with cDNA template prepared from cells suspecting of expressing the desired neurotrophin. The products of PCR can then be used as probes to permit cloning of complete cDNA and/or genomic genes, the sequences of which can be determined by standard methods. Novel

neurotrophins can be identified by selecting those containing, in addition to the sequences homologous to other known neurotrophins, sequences non-homologous to other known neurotrophins (e.g., at least six contiguous nucleotides in which at least two nucleotides differ). Similarly, oligonucleotides corresponding to sequences of a neurotrophin in one species can be used in PCR to generate probes to permit cloning of the neurotrophin gene from other species.

NGF and BDNF are basic proteins of approximately 120 amino acids that share about 50% amino acid sequence identity, including absolute conservation of six cysteine residues that, in active NGF, have been shown to form three disulfide bridges (Bradshaw, A., 1978, Ann. Rev. Biochem. 47:191-216; Leibrock et al., 1989, Nature 341:149-52). Comparison of the sequences of NGF from evolutionarily divergent species has revealed that the amino acids flanking these cysteine residues comprise the most highly conserved regions of the molecule (Meier et al., 1986, EMBO J. 5:1489-93; Selby et al., 1987, J. Neurosci. Res. 18:293-8). Strikingly, these are also the regions which are most similar between BDNF and NGF (Leibrock et al., 1989, Nature 341:149-52.

In a preferred aspect of the present invention, a mature human neurotrophin is produced by expression of a chimeric prepro molecule according to the present invention. In a specific embodiment, the chimeric prepro molecule is encoded by a nucleic acid containing the long prepro region of NGF fused in frame to the coding sequence for mature BDNF. In another embodiment, the chimeric prepro molecule is encoded by a nucleic acid containing the prepro region of NT-3 fused in frame to the coding region for mature

BDNF. In yet another embodiment, the long prepro region of NGF is fused in frame to the coding region for NT-3.

As discussed supra, no distinct biological significance between the "long" and "short" prepro region of the NGF precursor has been documented. In another specific aspect of the invention, either the "long" or "short" prepro region may be utilized in the construction of chimeric neurotrophic genes. One of ordinary skill in the art can utilize either a "short" NGF prepro region or a "long" NGF prepro region when constructing chimeric fusions of the present invention comprising an NGF prepro region.

The mature neurotrophin molecules which can be expressed as chimeric prepro precursors according to the present invention also include substantially equivalent sequences, and fragments or derivatives which are biologically active.

For example, the neurotrophin nucleic acid sequences can be altered by substitutions, additions or deletions that provide for functional molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences that encode substantially the same neurotrophin amino acid sequence may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the neurotrophin genes that are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the neurotrophin proteins, or fragments or derivatives thereof, of the invention include, but are not limited to, those containing, as part of their primary amino acid sequence, altered sequences in which functionally equivalent amino acid residues are substituted for

residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurotrophin proteins or fragments or derivatives thereof which are obtained through modification during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, acetylation, phosphorylation, reduction, cleavage, etc.

Additionally, a given neurotrophin sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

The present invention also relates to expression of the nucleic acids encoding chimeric prepro neurotrophin molecules, and recovery of the mature neurotrophin product.

5.2. THE CONSTRUCTION OF CHIMERIC NEUROTROPHIC PREPRO PROTEINS OR PREPRO PEPTIDES

Nucleic acids encoding chimeric neurotrophic prepro proteins or prepro peptides may be constructed using standard recombinant DNA technology, for example, by restriction enzyme digestion and ligation of nucleic acid sequences which encode the desired prepro and mature regions. Alternatively, nucleic acid sequences may be constructed using chemical synthesis, such as solid-phase phosphoramidate technology. In preferred embodiments of the invention, polymerase chain reaction (PCR; Saiki et al., 1985, Science 230:1350-1354) may be used to accomplish splicing of nucleic acid sequences by overlap extension (Horton et al., 1989, Gene 77:61-68) and thereby produce nucleic acids encoding the chimeric neurotrophic prepro proteins or prepro peptides of the invention (see e.g., Section 6, infra).

In a preferred aspect, the nucleic acids of the invention are produced by use of two separate PCR reactions, each with a different template. By way of illustration, if an X-Y chimera is desired, PCR is first carried out with one template, for example, X, using a probe completely homologous to X, and a probe with a region homologous to X and a region homologous to Y. The PCR reaction product is then isolated and used as probe in a second PCR reaction, with Y as a template, and a second probe completely homologous to Y.

It may further be desirable to incorporate useful restriction endonuclease cleavage sites in the primers.

In addition, chimeric neurotrophic factors may be produced by one-step PCR utilizing three oligonucleotide primers. For example, a nucleic acid encoding at least a portion of a desired prepro region (X) may be ligated to a nucleic acid sequence encoding a mature neurotrophic protein or peptide (Y) by creating three oligonucleotide primers, one of which corresponds to a portion of the X sequence (the "X primer"), another which corresponds to a portion of the Y sequence (the "Y primer"), and a third which contains a portion of both X and Y sequences ("the XY primer"). These three oligonucleotides may be combined in a one-step PCR, it being desirable that the X and Y primers are present in greater amounts than the XY primer, for example, at a ratio of X:XY:Y of about 100:1:100. [The template utilized in the PCR may be a mixture of nucleic acids encoding the desired prepro region and the mature neurotrophic protein or peptide.] The position of the splice site is determined by the bridging nucleotide (e.g. the XY primer).

Amplification conditions routinely used in the art may be used, for example, 1 minute at about 94°C, 2 minutes at about 43°C and 3 minutes at about 72°C for 35 cycles, using standard PCR reaction solutions and methods. The resulting PCR fragment may then be gel purified using gel electrophoresis, digested with the appropriate restriction endonuclease and ligated into a suitable cloning vector.

Additional methods of constructing the chimeras of the present invention will be readily apparent to those skilled in the art.

DNA reaction products may be cloned using any method known in the art. Any number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, pUC, or Bluescript® (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

5.3. EXPRESSION OF NUCLEIC ACIDS ENCODING CHIMERIC NEUROTROPHIC PREPRO PROTEINS OR PREPRO PEPTIDES

The nucleotide sequence coding for a chimeric neurotrophic prepro protein or prepro peptide, can be ligated into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription of the cloned chimeric DNA sequence. The necessary transcriptional and translation signals can also be supplied by one of the neurotrophin genes and/or its flanking regions corresponding to the chimeric neurotrophic prepro protein or prepro peptide. A variety of eukaryotic host-vector systems may be utilized to express the cloned chimeric DNA sequence and resulting mRNA transcript. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.), transfected with other vectors, containing chromosomally integrated nucleic acids of the invention, etc., but the host system used must have the appropriate cell machinery to process the prepro chimera to the mature neurotrophin. The expression elements of vectors vary

in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a sequence encoding a chimeric neurotrophic prepro protein or prepro peptide, consisting of appropriate transcriptional/translational control signals upstream of the chimeric DNA sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequences encoding chimeric neurotrophic prepro protein or prepro peptide may be regulated by a second nucleic acid sequence so that chimeric neurotrophic prepro protein or prepro peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression may be controlled by any promoter/enhancer element known in the art to be active in mammalian cells. Promoters which may be used to control chimeric neurotrophic factor expression include, but are not limited to, the cytomegalovirus (CMV) promoter, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic

animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

A specific example of an expression vector which can be used is CDM8 (Seed, 1987, Nature 329:840-842; Seed and Aruffo, 1987, Proc. Natl. Acad. Sci. USA 84:3365-3369; Aruffo & Seed, Proc. Natl. Acad. Sci.

USA 84: 8573-8577); another example being pCMX (see copending application Serial No. 678,408, filed March 28, 1991).

Expression vectors containing chimeric neurotrophic prepro protein or prepro peptide gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to at least a portion of an inserted chimeric neurotrophic prepro protein or prepro peptide gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, transformation phenotype, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric neurotrophic prepro protein or prepro peptide coding sequence is inserted within the marker gene sequence of the vector, recombinants containing the chimeric insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the neurotrophic factor gene product in bioassay systems as described infra, in Section 5.4.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established,

recombinant expression vectors can be propagated and prepared in quantity.

Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered chimeric neurotrophic prepro protein or prepro peptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems should be chosen to ensure the necessary processing (e.g., removal by cleavage of the prepro region) and any desired modification. Mammalian host cells, such as monkey, human, or bovine, are thus preferred.

In specific embodiments of the invention, DNA encoding chimeric neurotrophins may be expressed in a CHO cell system according to methods set forth infra. Once a recombinant which expresses the chimeric neurotrophin is identified, the mature gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product. See infra Section 5.4.

Once the mature neurotrophic factor protein or peptide is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

5.4. NEUROTROPHIC FACTOR ASSAYS

The neurotrophin proteins and peptides produced according to the invention are able to exhibit one or more biological activities including

but not limited to neurotrophic activity, binding by antibodies to neurotrophins, binding to cognate receptors, etc. The term "neurotrophic activity", as used herein, should be construed to refer to a biological effect on nervous system cells, including, but not limited to, neurons, astrocytes, glial cells, oligodendrocytes, microglia and Schwann cells. The biological effect is an alteration in the structure and/or physiology of a nervous system cell which does not occur absent direct or indirect exposure to the chimeric neurotrophic factor. Examples of a biological effects are the prolongation of survival, neurite sprouting, the maintenance or development of differentiated functions (such as expression of an enzyme e.g. choline acetyltransferase or tyrosine hydroxylase) or, conversely, cell death or senescence, or dedifferentiation.

The presence of neurotrophic activity may be determined using any known assay for such activity as well as systems which may be developed in the future. Assay systems may include in vitro testing systems, such as tissue culture bioassay systems using tissue explants, cells prepared from tissue, or immortalized cell lines, for example, derived from the brain, spinal cord, or peripheral nervous system, as well as in vivo testing systems in which neurotrophic factor may be administered to an animal; neurotrophic effects may be detected in such an animal by performing, chemical, histologic, or behavioral tests using said animal. Additionally, a neurotrophic factor may be incorporated as a transgene in a non-human transgenic animal, and its biological effects may be measured in said animal.

For example, but not by way of limitation, neurotrophic activity may be measured using any of the following well known bioassay systems:

(i) dorsal root ganglia assay system, as described in Barde et al., 1980, Proc. Natl. Acad. Sci. USA. 77:1199-1203, which is incorporated by reference in its entirety herein;

(ii) nodose ganglia assay system as described by Lindsay et al., 1985, Dev. Biol. 112:319-328, which is incorporated by reference in its entirety herein;

(iii) sympathetic ganglia assay as described in Barde et al., 1982, EMBO J. 1:549-553, which is incorporated by reference in its entirety herein;

(iv) spinal cord neurons. Briefly, spinal cords may be removed aseptically from a test animal, severed caudal to the bulb, and freed of sensory ganglia and meninges. The cord may then be subdivided into ventral and mediodorsal segments for separate cultures, and the tissues minced into small pieces and dissociated by trituration through a Pasteur pipet in 50 percent DMEM (Gibco) and 50 percent Ham's nutrient mixture F12 (Gibco) supplemented with 33 mM glucose, 2 mM glutamine, 15 mM $NaHCO_3$, 10 mM HEPES, 25 µg/ml insulin, 100 µg/ml transferrin, 60 µm putrescine, 20 nM progesterone, 30 nM Na selenite, 0.5 µg/ml penicillin G, 0.5 µg/nl streptomycin, and 2.5 µg/ml bovine serum albumin. Trituration may then be repeated twice and supernatants may be pooled and filtered through a 40 µm

Tetko filter. Dissociated ventral cells may then be plated in on poly-D-lysine coated (10 μg/ml) culture dish at a density of 0.5 million cells per 35 mm dish. Dissociated mediodorsal cells may be plated at a density of 1.5 million cells per 35 mm dish coated with poly-D-lysine (10 μg/ml), poly-L-ornithine (10μg/ml) or poly-L-ornithine plus laminin (5μg/ml).

(v) basal forebrain cholinergic neuron assays (see PCT Publication WO 91/03568, published March 21, 1991);

(vi) ventral mesencephalic dopaminergic neuron assay (see PCT Publication WO 91/03568, published March 21, 1991); and

(vii) PC12 cell assays.

6. EXAMPLE: CONSTRUCTION AND EXPRESSION OF THE PREPRO-NGF/MATURE-BDNF CHIMERA

6.1. CONSTRUCTION OF CHIMERIC NUCLEIC ACID MOLECULES USING POLYMERASE CHAIN REACTION

A polymerase chain reaction cloning was utilized (PCR; Saiki et al., 1985, Science 230: 1350-1354) to construct a prepro NGF/mature BDNF chimera consisting of the long prepro form of mouse NGF fused to the mature human BDNF sequence.

To accomplish this, two PCR primers were synthesized. The 5' primer (5'- CTC-GTC-GAC-AGC-CGG-CAC-TCT-GAC-CCT-GCG-CGC-CGA-3') [SEQ ID NO:17] encoded the first 7 amino acids of BDNF and included two unique restriction sites, NaeI and BssH2 which were generated by modifying codon usage. The 3' PCR primer was a 3' pCDM8 oligo corresponding to a region

downstream from the polylinker sequence at the 3' end of the BDNF sequence in pC8hB (5'-CAA-AGA-TCC-TCT-AGA-GTC-G-(C)-3') [SEQ ID NO:18]. The polylinker contains a Not1 restriction site. These two primers were used in PCR with pC8hB (hBDNF in pCDM8) DNA as template. 5 micrograms of pC8hB was used with 500 ng of each primer for 5 PCR cycles. The PCR product was digested with both Nae1 and Not1 simultaneously and a 365 bp digestion product was isolated by gel electrophoresis. The preparation of the vector was carried out by digesting pC81mN (long mouse NGF in pCDM8) with both Eco47 and Not1 and isolating the 4.6 kb vector fragment by gel electrophoresis. The 365 bp fragment was ligated into the Eco47/Not1 sites of pC81mN. This ligation resulted in a direct in frame fusion of the mouse NGF prepro region with the mature BDNF coding region. Constructs were diagnostically tested by digesting with BssH2, by assessing the loss of the Eco47 site during the subcloning, and ultimately by DNA sequencing.

6.2. EXPRESSION OF CHIMERIC MOLECULES

CHO-DG44 cells were used to generate stable lines for the production of bioactive BDNF. CHO-DG44 cells (obtained from Dr. L. Chasin at Columbia University) lack both copies of the dihydrofolate reductase gene (Urlaub and Chasin, 1980, Proc. Natl. Acad. Sci. USA 77:4216-4220). Stably transfected CHO-DG44 cell lines expressing BDNF have been previously described (PCT International Publication No. WO 91/03568, published March 21, 1991). These lines were generated by transfection with pC8hB DNA which encodes the human BDNF gene including the prepro region cloned into the expression vector pCDM8. CHO-DG44 cells (1 x 10^6 cells/100 mm plate) were

transfected by the calcium phosphate coprecipitation method with 20 μg of the NGF/BDNF chimera (pC81mN/B) along with 0.2 μg of plasmid p410 which encodes a weakened dihydrofolate reductase gene (dhfr). 48 hours after transfection, the cells were passaged into selection media (Ham's F12 without hypoxanthine and thymidine containing 10% dialyzed fetal bovine serum and 1% each of penicillin and streptomycin; -HT media). -HT-resistant clones were treated as pools for amplification with methotrexate (MTX). Clones obtained with 0.05 μM MTX were also treated as pools for further amplification at 2.5 μM MTX. A single clone that was selected first in 0.5 μM MTX and then in 2.5 μM MTX (thus 2 rounds of amplification) was isolated (DGC-N/B-2.5-#23) which proved to be the highest producer of BDNF as assessed by both bioactivity and metabolic labeling. Bioactivity was assessed by scoring neurite outgrowth of embryonic (E8) chick dorsal root ganglia (DRG) (Maisonpierre et al., 1990, Science 247:1446-1451).

7. EXAMPLE: COMPARISON OF PROCESSING EFFICIENCY BETWEEN HOMOLOGOUS PREPRO BDNF AND PREPRO NGF/BDNF CHIMERA

Experiments were performed to directly compare the processing and expression of preproBDNF with the preproNGF/BDNF chimera in CHO cells.

7.1. METABOLIC LABELING

CHO-DG44 cell lines stably transfected with either pC8hB or pC81mN/B and amplified with 2.5 μM methotrexate were compared by metabolic labeling (Figure 1). For this labeling experiment, CHO-DG44 cells expressing BDNF from either the short prepro BDNF construct (cell line = DGZ1000-B-3-2.5) or the long mouse preproNGF/BDNF chimeric construct (cell

line = DGC-N/B-2.5-#23) were seeded at equal densities (2 x 10^5 cells/well in 6-well plate) 24 hours prior to labeling. The cells were then labeled with both ^{35}S-cysteine and ^{35}S-methionine for 4 hours under serum-free conditions. 30 µl aliquots of labeled cell supernatants were resolved by SDS polyacrylamide gel electrophoresis (15% gel) and labeled proteins were transferred to nylon membranes and visualized by autoradiography. As observed in Figure 1, CHO-DG44 cells stably transfected with the human NGF gene in the expression vector pCDM8 expressed the mature form of NGF migrating at a molecular weight of approximately 12,300 (Figure 1, lane 2). Wild type CHO-DG44 cells as control are shown in lane 1. Unprocessed proBDNF (31 kD), the pro-portion of the processed proBDNF precursor (16 kD) and the mature form (14 kD) of the short preproBDNF protein were detected in the stably transfected cell line DGZ1000-B-3-2.5 (obtained after similar MTX selection and amplification as used for cell line DGC-N/B-2.5-#23) (Figure 1, lane 3). Only the proteolytically processed mature form of BDNF (14 kD) was detected in DGC-N/B-2.5-#23, stably transfected with the long proNGF/BDNF chimeric construction (Figure 1, lane 4). Unprocessed proNGF/BDNF was not detected in the conditioned media from this cell line. We estimate from the intensity of the labeling of the mature BDNF that cell line DGC-N/B-2.5-#23 produced about five (5) times as much mature BDNF protein per cell relative to cell line DGZ1000-B-3-2.5 made with the short proBDNF construct.

7.2 BIOACTIVITY

The bioactivity of BDNF produced in the two CHO cell lines described above were compared. Crude supernatants were assayed with embryonic (E8) chick dorsal root ganglia and neurite outgrowth was scored. Consistent with the metabolic labeling experiments, the cell line DGC-N/B-2.5-#23 appeared to produce approximately five (5) times as much mature BDNF relative to cell line DGZ1000-B-3-2.5. For example, maximal neurite outgrowth was achieved with 10 μl of supernatant derived from DGC-N/B-2.5-#23 cells while 50 μl of DGZ1000-B-3-2.5 supernatant was required to achieve maximal DRG bioactivity (Figure 2).

7.3. COMPARISON OF EXPRESSION OF NGF USING LONG AND SHORT NGF PREPRO REGIONS

COS cells were transfected with prepro NGF containing either the long ("lmNGF") or short ("smNGF") NGF prepro region with the mature NGF coding region. Culture supernatants were harvested 48 hours after transfection and assayed on DRG explants, along with purified NGF and a mock transfected COS cell supernatant. Results using three different concentrations of each construct, as shown in Table 1, reveal significant bioactivity of NGF expressed with either the long or short form of the prepro region.

TABLE 1
Effect of Various COS
Supernatants on DRG Explants

SAMPLE	DILUTION	DRG
(-) CONTROL NGF	 10 ng/ml	0,0,0,0,0.5 5+,5+,5+,5+,5+
MOCK	10 µl	0,1,1,1,1
	50 µl	0.5,1,1,1,1.5
	100 µl	0.5,0.5,1,1,1
	250 µl	2,2.5,2.5,2.5,2.5
smNGF	10 µl	2,3,3,3,3.5
	50 µl	5,5,5,5,5
	100 µl	5+,5+,5+,5+,5+
	250 µl	5+,5+,5+,5+,5+
lmNGF	10 µl	2,2,2,2,2
	50 µl	4,4,4,4,4
	100 µl	5,5,5,5,5
	250 µl	5,5,5,5,5

7.4. CONCLUSIONS

We conclude from these studies that the long pro portion of NGF is better suited for the processing of BDNF in CHO cells than the short pro portion of BDNF. The advantages of the chimeric proNGF/mature BDNF gene construct, therefore, is that it allows for higher expression levels of BDNF on a per cell basis in mammalian cells. Additionally, it should allow for better purification schemes for BDNF in that contaminating unprocessed forms of BDNF are not apparent in the crude supernatants.

Additionally, use of either the long or short prepro region of NGF results in the expression of biologically active NGF. This indicates that either the long or short prepro region of NGF may be utilized in the construction of chimeric neurotrophic genes.

8. EXAMPLE: CONSTRUCTION AND EXPRESSION OF THE PREPRO-NT-3/BDNF CHIMERA

8.1. CONSTRUCTION OF CHIMERIC NUCLEIC ACID MOLECULES

A HindIII-Xho1 DNA fragment containing the entire coding region of prepro and mature human BDNF was obtained from digestion of plasmid pC8hB with corresponding restriction enzymes. The plasmid pC8hB was derived by cloning the human BDNF coding sequences, including the entire prepro region, into the expression vector pCDM8 (discussed supra). This fragment was ligated to pDSRα2 (see published European patent application 90305433.6 EPO Publication No. 0398753A2, incorporated herein by reference in its entirety. The plasmid pDSRα2 had been previously digested to make available the cloning sites 5'-HindIII and 3'-Sal1 for ligation of the human BDNF

containing fragment. The resulting plasmid was designated pDSRα2(BDNF).

For generating a chimeric plasmid with a prepro NT-3 sequence and a mature BDNF sequence, three DNA fragments were prepared as follows and then ligated in a specific orientation. An approximately 400-bp 5'-HindIII/3'-NarI DNA fragment containing all of the prepro human BDNF sequence was deleted by restriction enzyme digestion from the expression plasmid pDSRα2(BDNF) described above. A DNA fragment recovered from this digestion contained the entire expression vector pDSRα2 and the mature human BDNF sequence, bordered by the 5'-HindIII and a 3'-NarI sites (labeled DNA fragment No. 1). An approximately 300-base pair 5'-HindIII/3'-SacII DNA fragment containing the prepro region of human NT-3 was obtained through digestion of plasmid pC8hN3 with corresponding restriction enzymes. Coding sequences corresponding to 35 amino acid residues of the prepro NT-3 region were deleted downstream of the SacII site as a consequence of the digestion. The plasmid pC8hN3 was derived by cloning the human NT-3 coding sequences, including the entire prepro region, into the expression vector pCDM8. The 300-base pair 5'-HindIII/3'-SacII fragment was labeled DNA fragment No. 2. Finally, DNA fragment No. 3 was prepared, which was an oligonucleotide linker synthesized to regenerate the aforementioned missing 35 amino acid residues (Figure 6 and SEQ ID NO:15-17). The linker also contained the half sites of the 5'-SacII and 3'-Nar1 restriction sites to promote ligation to DNA fragments Nos. 1 and 2 disclosed supra. This ligation resulted in the expression vector pDSRα2(NT-3/BDNF), in which the prepro region of NT-3 (fragment No. 2) is joined with mature BDNF (fragment No. 1) by the

oligonucleotide linker (fragment No. 3; Figure 6 and SEQ ID NO:15).

8.2. EXPRESSION AND CHARACTERIZATION OF NT-3/BDNF CHIMERA IN CHO CELLS

CHO-D(-) cells (ATCC accession number CCL 61) were used to generate stable lines for the production of bioactive BDNF. CHO-D(-) cells are defective in the gene encoding dihydrofolate reductase and are maintained in the medium of Dulbecco's modified Eagle media (D-MEM), supplemented with MEM nonessential amino acids, 1% each of penicillin and streptomycin, 10% fetal bovine serum, hypoxanthine and thymidine. CHO-D(-) cells (0.8 x 10^6/60 mm plate) were transfected by the calcium phosphate coprecipitation method, using 2.5 μg of the NT-3/BDNF chimeric construction [pDSRα2(NT-3/BDNF)] previously linearized by digestion with restriction enzyme PvuI. This vector (pDSRα2) encodes a mouse dihydrofolate reductase minigene (dhfr) which, when expressed, enables the transfected CHO-D(-) to overcome the deficiency of the dhfr gene and become capable of growing in the absence of the nucleotides hypoxanthine and thymidine. Parental CHO-D(-) cells or cells not successfully transfected by the vector pDSRα2 will not survive in the selection media, which has the composition of the maintenance media described above except that fetal bovine serum is substituted with dialyzed fetal bovine serum and hypoxanthine and thymidine are omitted. The cells were trypsinized and seeded 48 hours after transfection at 1 x 10^5 cells/100 mm plate in selection media. Individual colonies were picked two weeks later using cloning cylinders. Each clone was then expanded to 100 mm plates. When the cultures reached confluency, the original serum-

containing media were aspirated and replaced with 3 ml of serum free media. The conditioned media (CM) were collected and 50 µl each was loaded on a 15% SDS-polyacrylamide gel and subjected to gel electrophoresis. Western blotting of the gel was performed with rabbit antiserum specific for mature BDNF. As shown in Figure 7, all clones expressing the original BDNF from pDSRα2(BDNF) secreted multiple forms of unprocessed BDNF, in addition to the mature, processed BDNF. The ratio of unprocessed forms to processed form was about 2:1. In contrast, all of the clones expressing chimeric NT-3/BDNF from pDSRα2(BDNF) secreted only the fully processed, mature form of BDNF with no detectable partially processed precursors.

One liter of serum-free conditioned media from one of the chimeric NT-3/BDNF clones was subjected to purification by passage through an S-Sepharose column followed by a Sephacryl S-200 size exclusion column. SDS-PAGE analysis and amino acid sequence determination showed that a homogeneous protein with a molecular weight of 14 kd (as predicted for mature human BDNF) was obtained, with a unique N-terminal sequence in agreement with the N-terminal sequence of mature human BDNF. Furthermore, the purified BDNF was demonstrated by the chick dorsal root ganglia assay (described for the NGF/BDNF chimera, supra) to possess full biological activity.

8.3. EXPRESSION IN COS CELLS AND BIOACTIVITY OF THE NT-3/BDNF CHIMERA

COS-7 cells (ATCC accession number CRL 1651) were used as a transient expression system to test the production of bioactive BDNF. COS-7 cells are routinely maintained in D-MEM with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics.

COS-7 cells (5 x 10^6 cells/ml) were transfected by electroporation at 1600 volts for 0.4 msec with, individually, 20 μg each of pDSRα2, pDSRα2(BDNF) and pDSRα2(NT-3/BDNF). Transfected COS-7 cells were plated at 2 x 10^6 cells/60 mm plate. Conditioned medium accumulated between 24 and 72 hours post transfection was collected. Bioactivity was assessed by scoring neurite outgrowth of embryonic (E8) chick dorsal root ganglia (as with the NGF/BDNF chimera). As shown in Table 2, the clonal isolates CI 1 and CI 20 of chimeric pDSRα2(NT-3/BDNF) were approximately 5 times more active than mature BDNF expressed from pDSRα2(BDNF), the latter containing the unaltered prepro region of BDNF.

TABLE 2
Chick DRG Explant Assay
of Conditional Media from COS Cells
Transfected with Plasmid DNA

DNA Source	Volume of Medium Tested μL	Score of Neurite Outgrowth
pDSRα2	10 50	0,0,0,0,0 0,0,0,0.5,0.5
pDSRα2(BDNF)	10 50	0,0.5,0.5,0.5,0.5 1,1,1,1.5,1.5
pDSRα2(NT-3/BDNF), CI 2	10 50	1,1,1.5,1.5,1.5 2.5,2.5,2.5,2,2
pDSRα2(NT-3/BDNF), CI 20	10 50	1,1,2,2,2 2.5,2.5,2.5,2.5,2.5

8.4 CONCLUSIONS

These studies demonstrate that the substitution of the prepro region of BDNF with the NT-3 prepro region facilitates the proteolytic processing of the prepro region and significantly increases the net yield of mature BDNF. Further, the reconstituted cleavage site between the prepro NT-3 and mature BDNF DNA sequences was recognized accurately by the host cell without any alteration at the NH_2-terminus of the mature, processed BDNF. As with the chimeric NGF/BDNF gene construct, the chimeric NT-3/BDNF gene construct results for higher levels of processed BDNF on a per cell basis in mammalian cells, and it should also allow for better purification schemes by elimination or minimization of contaminating unprocessed forms.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in

addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications and patent applications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A chimeric prepro protein or prepro peptide comprising (a) a prepro region of a first neurotrophin; and (b) an amino acid sequence substantially equivalent to the mature form of a second neurotrophin, in which the first and second neurotrophins are different.

2. A chimeric prepro protein or prepro peptide comprising (a) prepro region of a first neurotrophin; and (b) a biologically active amino acid sequence substantially equivalent to a portion of the mature form of a second neurotrophin, in which the first and second neurotrophins are different.

3. The chimeric prepro protein of claim 1 in which the first and second neurotrophins are selected from the group consisting of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3.

4. The chimeric prepro protein of claim 2 in which the first and second neurotrophins are selected from the group consisting of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3.

5. The chimeric prepro protein or prepro peptide of claim 1 in which the prepro region is the long prepro region of nerve growth factor.

6. The chimeric prepro protein or prepro peptide of claim 2 in which the prepro region is the long prepro region of nerve growth factor.

7. The chimeric prepro protein or prepro peptide of claim 1 in which the prepro region is the short prepro region of nerve growth factor.

8. The chimeric prepro protein or prepro peptide of claim 2 in which the prepro region is the short prepro region of nerve growth factor.

9. The chimeric prepro protein or prepro peptide of claim 1 in which the first neurotrophin is neurotrophin-3.

10. The chimeric prepro protein or prepro peptide of claim 2 in which the first neurotrophin is neurotrophin-3.

11. The chimeric prepro protein or prepro peptide of claim 1 in which the first neurotrophin is brain-derived neurotrophic factor.

12. The chimeric prepro protein or prepro peptide of claim 2 in which the first neurotrophin is brain-derived neurotrophic factor.

13. The chimeric prepro protein or prepro peptide of claim 5 or 6 in which the second neurotrophin is brain-derived neurotrophic factor.

14. The chimeric prepro protein or prepro peptide of claim 7 or 8 in which the second neurotrophin is brain-derived neurotrophic factor.

15. The chimeric prepro protein or prepro peptide of claim 9 or 10 in which the second neurotrophin is brain-derived neurotrophic factor.

16. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 1.

17. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 2.

18. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 3.

19. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 4.

20. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 5.

21. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 6.

22. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 7.

23. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 8.

24. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 9.

25. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 10.

26. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 11.

27. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 12.

28. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 13.

29. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 14.

30. A nucleic acid molecule comprising a nucleotide sequence encoding the chimeric prepro protein or prepro peptide of claim 15.

31. The nucleic acid molecule of claim 16 or 17, which is a vector.

32. The nucleic acid molecule of claim 18 or 19, which is a vector.

33. A recombinant cell containing the vector of claim 31.

34. A recombinant cell containing the vector of claim 32.

35. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 1, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

36. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 2, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

37. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 3, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

38. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 4, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

39. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 5, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

40. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 6, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

41. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 7, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

42. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 8, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

43. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 9, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

44. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 10, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

45. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 11, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

46. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 12, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the portion of the mature form of the second neurotrophin.

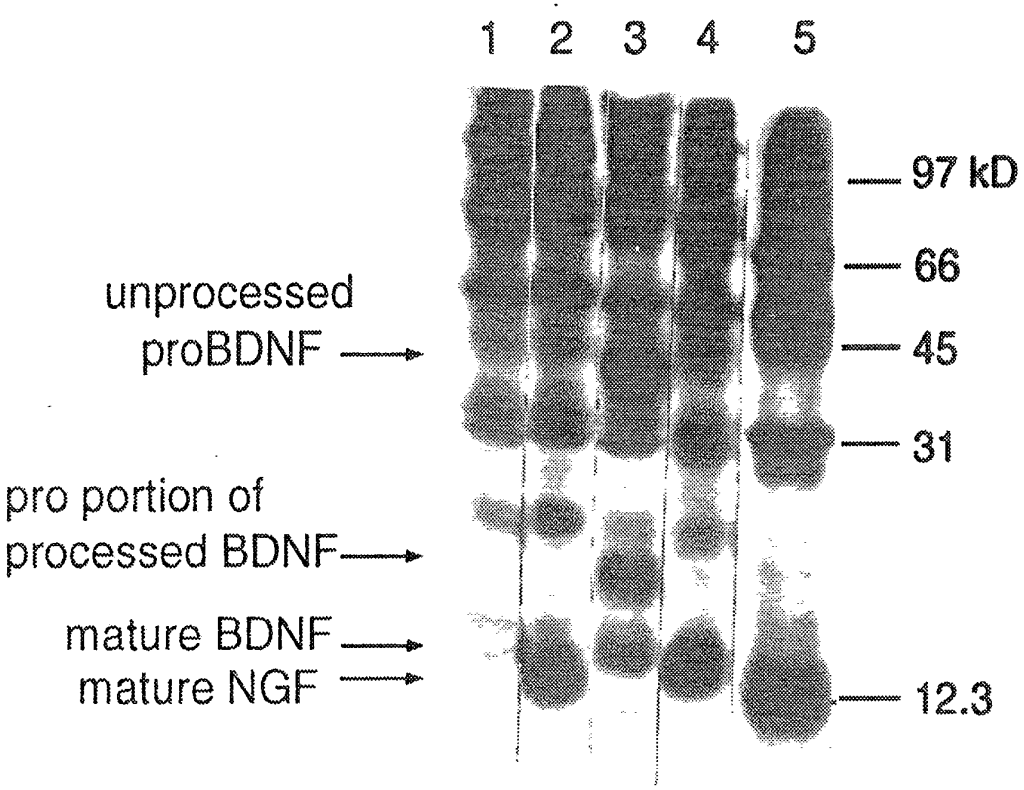
47. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 13, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

48. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 14, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

49. A method of producing a neurotrophin comprising growing a recombinant cell containing the nucleic acid molecule of claim 15, under conditions such that the chimeric prepro protein or peptide is expressed and processed by the cell to produce the mature form of the second neurotrophin.

50. The method according to claim 35 or 36 in which the produced mature form or portion thereof of the second neurotrophin is capable of exhibiting neurotrophic activity.

FIG. 1


1 2 3 4 5
97 kD
66
45
31
12.3
unprocessed
proBDNF
pro portion of
processed BDNF
mature BDNF
mature NGF

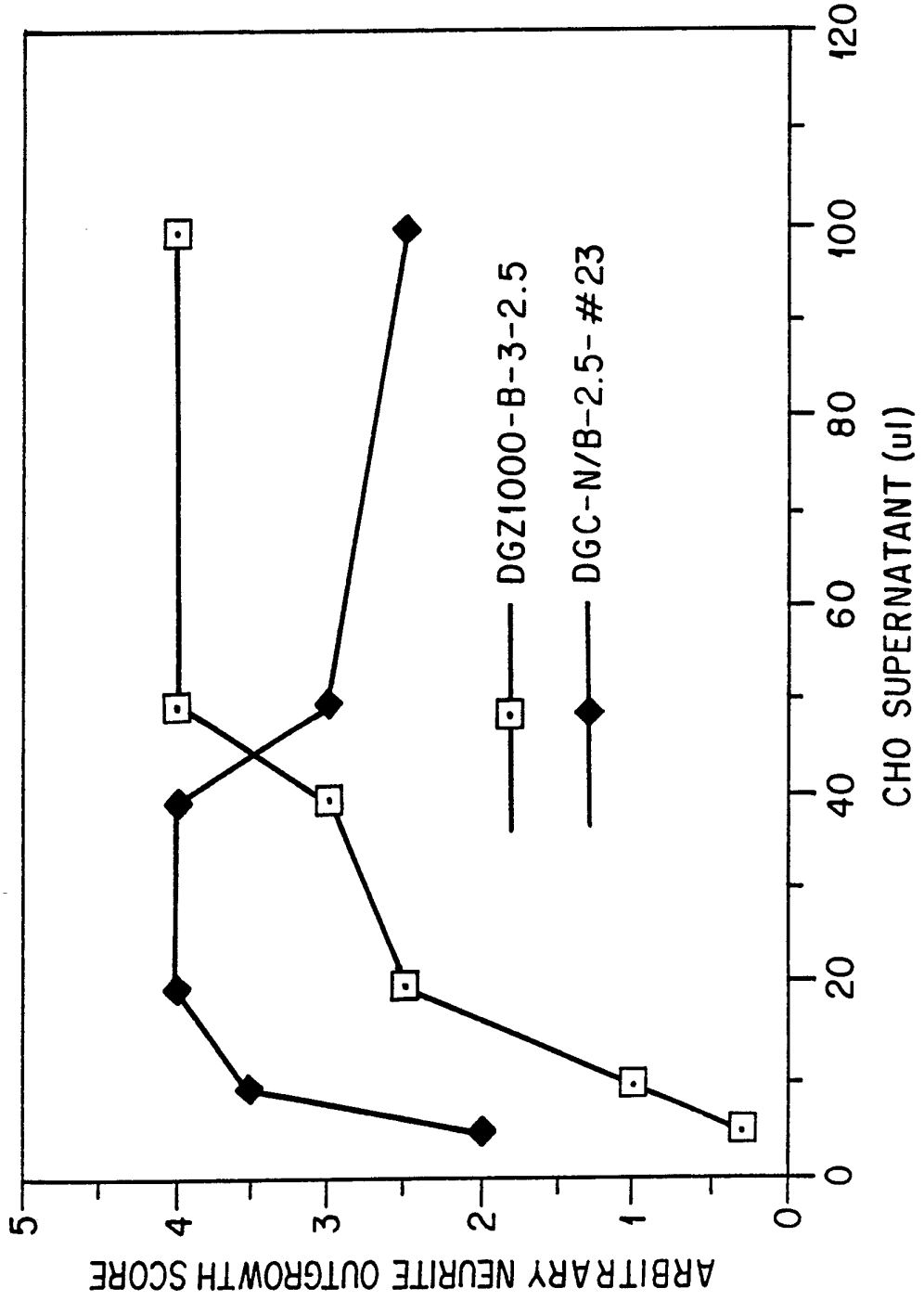

ARBITRARY NEURITE OUTGROWTH SCORE
0
1
2
3
4
5
0
20
40
60
80
100
120
CHO SUPERNATANT (ul)
DGZ1000-B-3-2.5
DGC-N/B-2.5-#23

FIG. 2

HBDNF
---100+---200+---300+---400+---500+---600+---700+---800+---900+---1000+---1100+---1200+---1300+---1400+---1500+---1600+-
PRE-PRO FORM OF BDN
MATURE FORM OF BDNF
RBDNF (3724)
RBDNF (3616)
CKBDNF (414)

FIG. 3

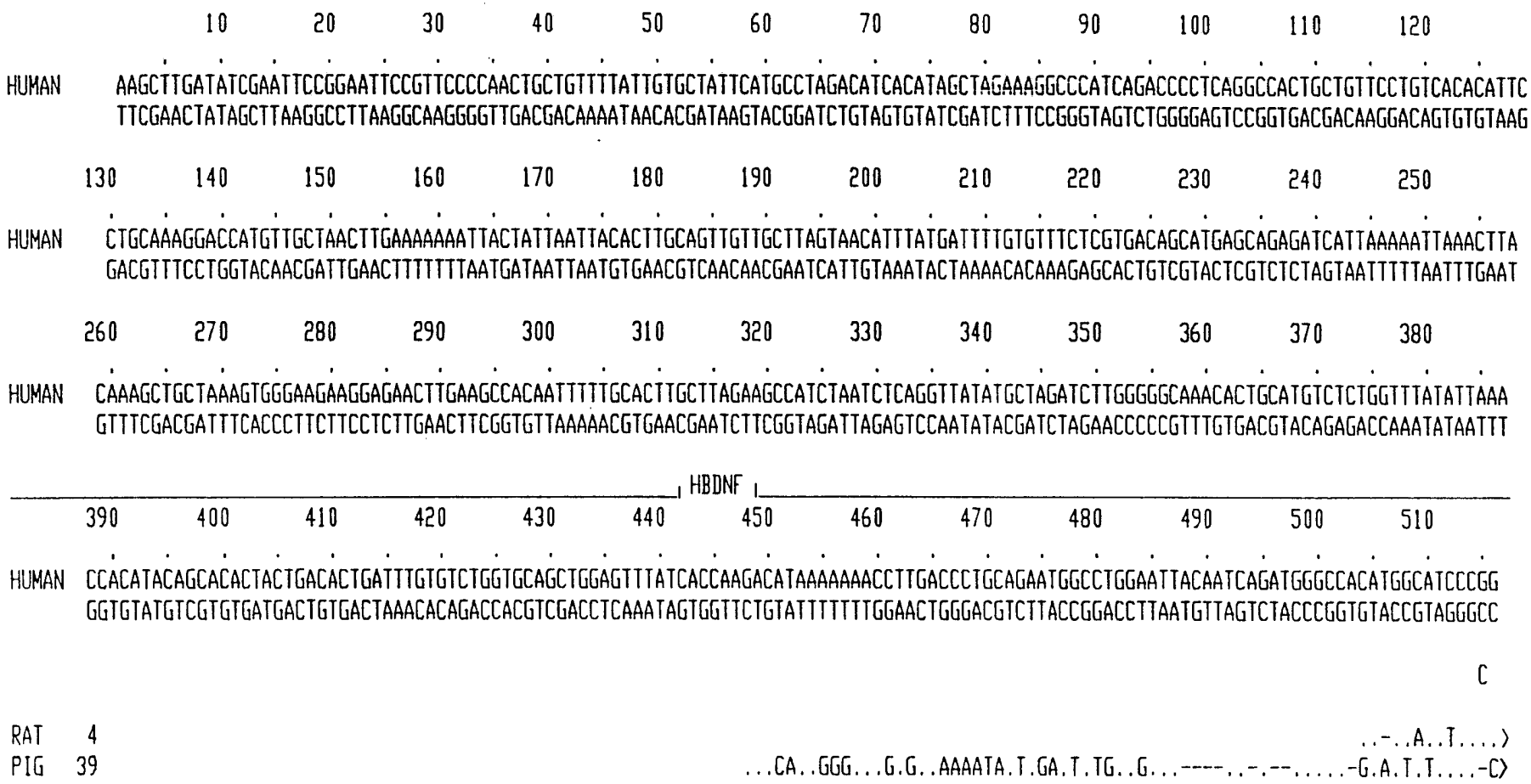

10 20 30 40 50 60 70 80 90 100 110 120
HUMAN AAGCTTGATATCGAATTCCGGAATTCCGTTCCCCAACTGCTGTTTTATTGTGCTATTCATGCCTAGACATCACATAGCTAGAAAGGCCCATCAGACCCCTCAGGCCACTGCTGTTCCTGTCACACATTC
TTCGAACTATAGCTTAAGGCCTTAAGGCAAGGGGTTGACGACAAAATAACACGATAAGTACGGATCTGTAGTGTATCGATCTTTCCGGGTAGTCTGGGGAGTCCGGTGACGACAAGGACAGTGTGTAAG
130 140 150 160 170 180 190 200 210 220 230 240 250
HUMAN CTGCAAAGGACCATGTTGCTAACTTGAAAAAAATTACTATTAATTACACTTGCAGTTGTTGCTTAGTAACATTTATGATTTTGTGTTTCTCGTGACAGCATGAGCAGAGATCATTAAAAATTAAACTTA
GACGTTTCCTGGTACAACGATTGAACTTTTTTTAATGATAATTAATGTGAACGTCAACAACGAATCATTGTAAATACTAAAACACAAAGAGCACTGTCGTACTCGTCTCTAGTAATTTTTAATTTGAAT
260 270 280 290 300 310 320 330 340 350 360 370 380
HUMAN CAAAGCTGCTAAAGTGGGAAGAAGGAGAACTTGAAGCCACAATTTTTGCACTTGCTTAGAAGCCATCTAATCTCAGGTTATATGCTAGATCTTGGGGGCAAACACTGCATGTCTCTGGTTTATATTAAA
GTTTCGACGATTTCACCCTTCTTCCTCTTGAACTTCGGTGTTAAAAACGTGAACGAATCTTCGGTAGATTAGAGTCCAATATACGATCTAGAACCCCCGTTTGTGACGTACAGAGACCAAATATAATTT
HBDNF
390 400 410 420 430 440 450 460 470 480 490 500 510
HUMAN CCACATACAGCACACTACTGACACTGATTTGTGTCTGGTGCAGCTGGAGTTTATCACCAAGACATAAAAAAACCTTGACCCTGCAGAATGGCCTGGAATTACAATCAGATGGGCCACATGGCATCCCGG
GGTGTATGTCGTGTGATGACTGTGACTAAACACAGACCACGTCGACCTCAAATAGTGGTTCTGTATTTTTTTGGAACTGGGACGTCTTACCGGACCTTAATGTTAGTCTACCCGGTGTACCGTAGGGCC
C
RAT 4 ..-..A..T....)
PIG 39 ...CA..GGG...G.G..AAAATA.T.GA.T.TG..G...----..-.--.....-G.A.T.T....-C)

FIG. 3A

```
                                                                  |HBDNF|
HUMAN   520       530       540       550       560       570       580       590       600       610       620       630
        .         .         .         .         .         .         .         .         .         .         .         .
        TGAAAGAAAGCCCTAACCAGTTTTCTGTCTTGTTTCTGCTTTCTCCCTACAGTTCCACCAGGTGAGAAGAGTG ATG ACC ATC CTT TTC CTT ACT ATG GTT ATT TCA TAC TTT GGT
        ACTTTCTTTCGGGATTGGTCAAAAGACAGAACAAAGACGAAAGAGGGATGTCAAGGTGGTCCACTCTTCTCAC TAC TGG TAG GAA AAG GAA TGA TAC CAA TAA AGT ATG AAA CCA
                                                                                  Met Thr Ile Leu Phe Leu Thr Met Val Ile Ser Tyr Phe Gly>
                                                                                  ___a___a___a___a_PRE-PRO FORM OF BDNF__a___a___a___a___>

RAT  17 CCTTG..C..AG.-C.G.G.A...G.-C.GA.G.GG.-AG.A..T.A.C.......................... ... ... ... ... ... ... ... ... ... ... ... ... ..C ...>
PIG  99 A..GCAGCT...T.G.--T....A..-.-.GACAAG.AG.GA..-AAA.A.......................... ... ... ... ... ... ... ... ... ... ... ... ... ..C ...>
```

FIG. 3B

SUBSTITUTE SHEET

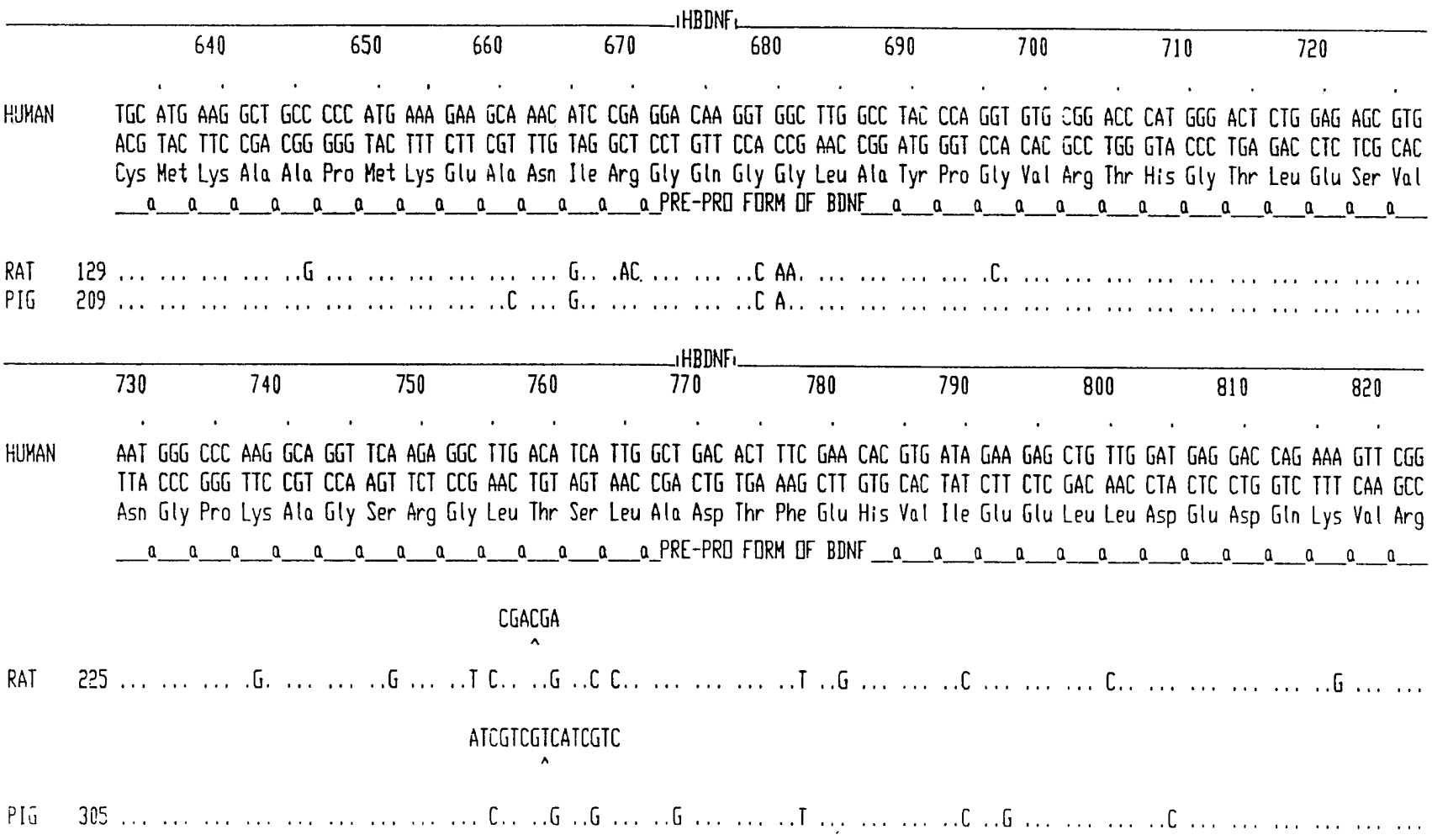


FIG. 3C

```
                                                  |HBDNF|
                 830       840       850       860       870       880       890       900       910
HUMAN     CCC AAT GAA GAA AAC AAT AAG GAC GCA GAC TTG TAC ACG TCC AGG GTG ATG CTC AGT AGT CAA GTG CCT TTG GAG CCT CCT CTT CTC TTT CTG CTG
          GGG TTA CTT CTT TTG TTA TTC CTG CGT CTG AAC ATG TGC AGG TCC CAC TAC GAG TCA TCA GTT CAC GGA AAC CTC GGA GGA GAA GAG AAA GAC GAC
          Pro Asn Glu Glu Asn Asn Lys Asp Ala Asp Leu Tyr Thr Ser Arg Val Met Leu Ser Ser Gln Val Pro Leu Glu Pro Pro Leu Leu Phe Leu Leu

          __a__a__a__a__a__a__a__a__a__a__a__a__a__a_PRE-PRO FORM OF BDNF__a__a__a__a__a__a__a__a__a__a__a__a__a__a__

RAT   327 ... ..C ... ... ... C.. ... ... ..G ... ... ... ..T ... C.. ... ... ... ..C ... ... ... ... ... ... ... ... ..G ... ... ... ...
PIG   416 ... ... ..G ... ... ... ... ... ..G ... A.. ..T ... ... C.A ..C ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ...
```

FIG. 3D

SUBSTITUTE SHEET

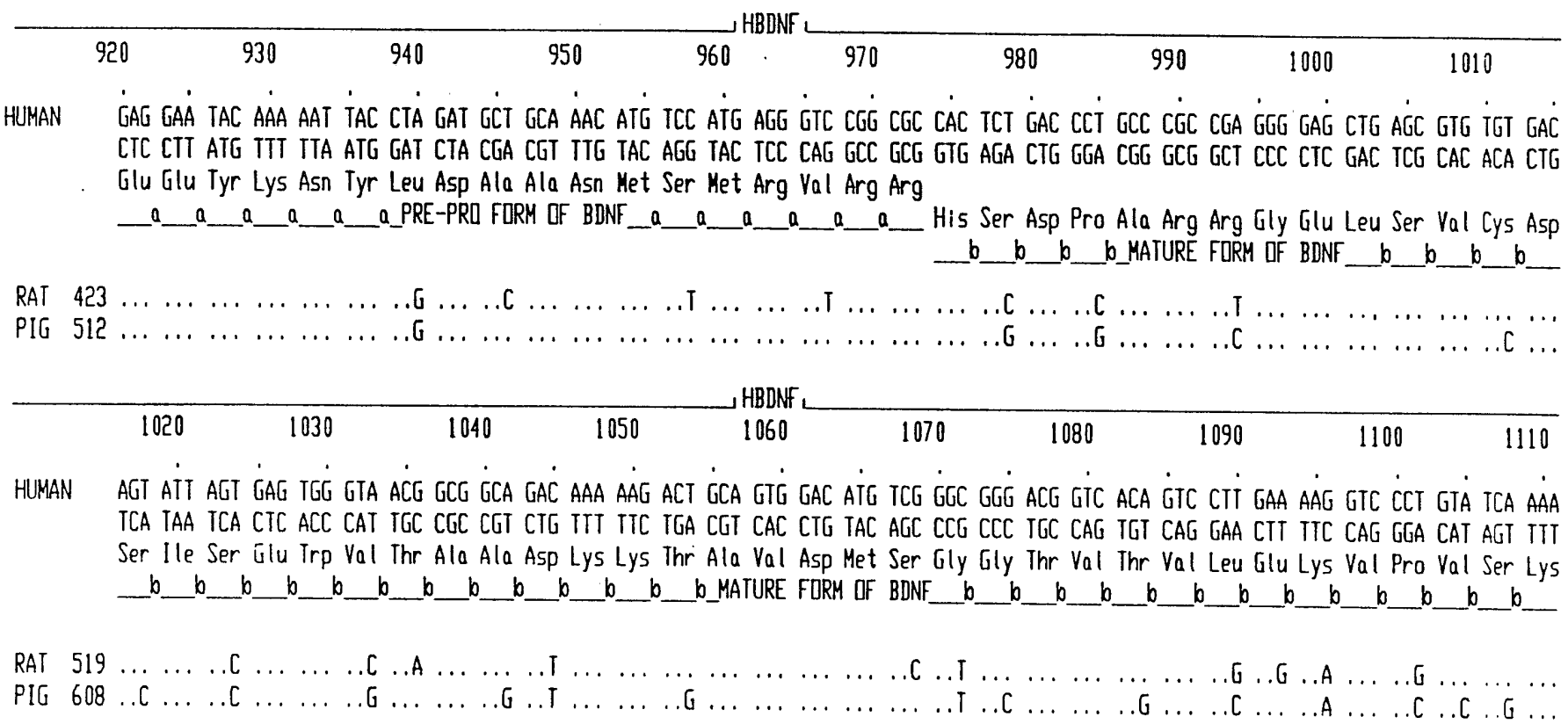


FIG. 3E

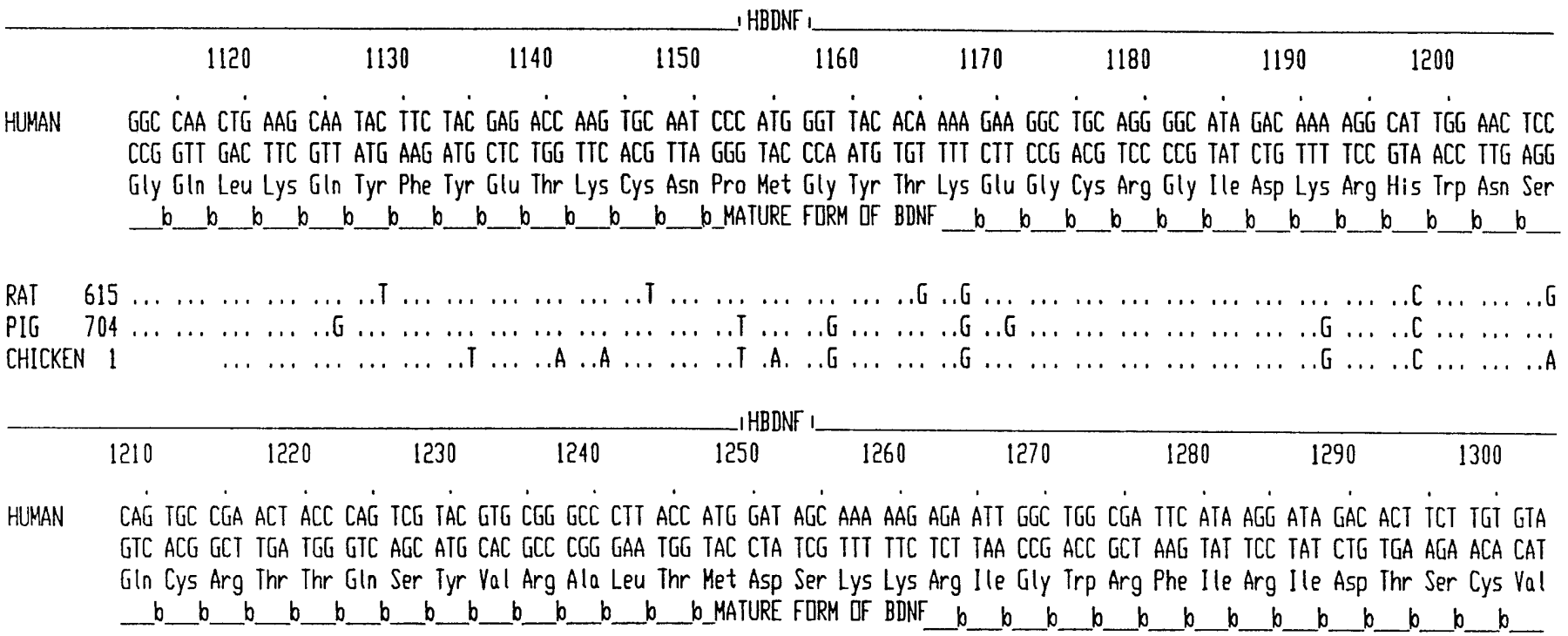

HBDNF
1120 1130 1140 1150 1160 1170 1180 1190 1200
HUMAN GGC CAA CTG AAG CAA TAC TTC TAC GAG ACC AAG TGC AAT CCC ATG GGT TAC ACA AAA GAA GGC TGC AGG GGC ATA GAC AAA AGG CAT TGG AAC TCC
CCG GTT GAC TTC GTT ATG AAG ATG CTC TGG TTC ACG TTA GGG TAC CCA ATG TGT TTT CTT CCG ACG TCC CCG TAT CTG TTT TCC GTA ACC TTG AGG
Gly Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser
MATURE FORM OF BDNF
RAT 615 ..T ..T ..G ..G ..C ..G
PIG 704 ..G ..T ..G ..G ..G ..G ..C ..C
CHICKEN 1 ..T ..A ..A ..T .A. ..G ..G ..G ..C ..A
HBDNF
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
HUMAN CAG TGC CGA ACT ACC CAG TCG TAC GTG CGG GCC CTT ACC ATG GAT AGC AAA AAG AGA ATT GGC TGG CGA TTC ATA AGG ATA GAC ACT TCT TGT GTA
GTC ACG GCT TGA TGG GTC AGC ATG CAC GCC CGG GAA TGG TAC CTA TCG TTT TTC TCT TAA CCG ACC GCT AAG TAT TCC TAT CTG TGA AGA ACA CAT
Gln Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val
MATURE FORM OF BDNF

FIG. 3F

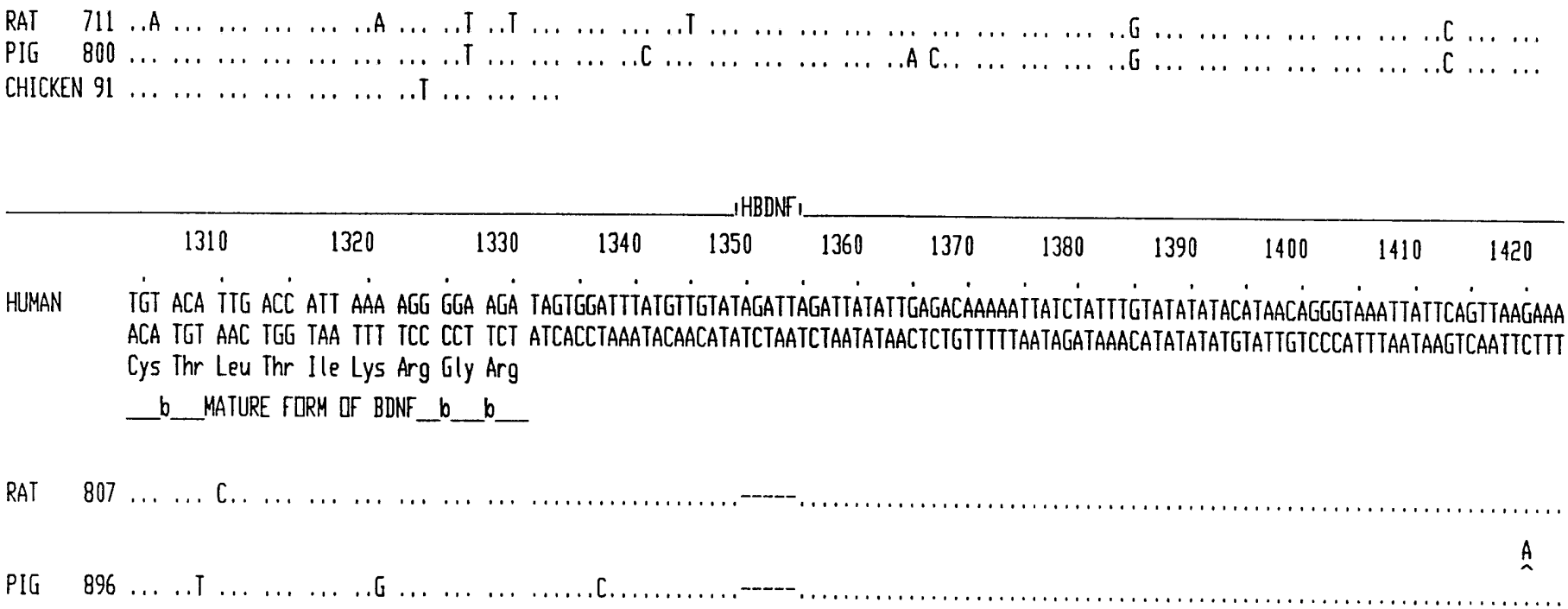

RAT 711 ..A ... ...A ... ..T ..T ... ...T ... ...G ... ...C ... ...
PIG 800 ... ...T ... ...C ... ...A C.. ... ...G ... ...C ... ...
CHICKEN 91 ... ...T ... ...
HBDNF
1310 1320 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420
HUMAN TGT ACA TTG ACC ATT AAA AGG GGA AGA TAGTGGATTTATGTTGTATAGATTAGATTATATTGAGACAAAAATTATCTATTTGTATATATACATAACAGGGTAAATTATTCAGTTAAGAAA
ACA TGT AAC TGG TAA TTT TCC CCT TCT ATCACCTAAATACAACATATCTAATCTAATATAACTCTGTTTTTAATAGATAAACATATATATGTATTGTCCCATTTAATAAGTCAATTCTTT
Cys Thr Leu Thr Ile Lys Arg Gly Arg
__b__MATURE FORM OF BDNF__b__b__
RAT 807 ... ... C.. ... ...-----.....
A
PIG 896 ... ..T ... ...G ... ...C.....-----.....

FIG. 3G

SUBSTITUTE SHEET

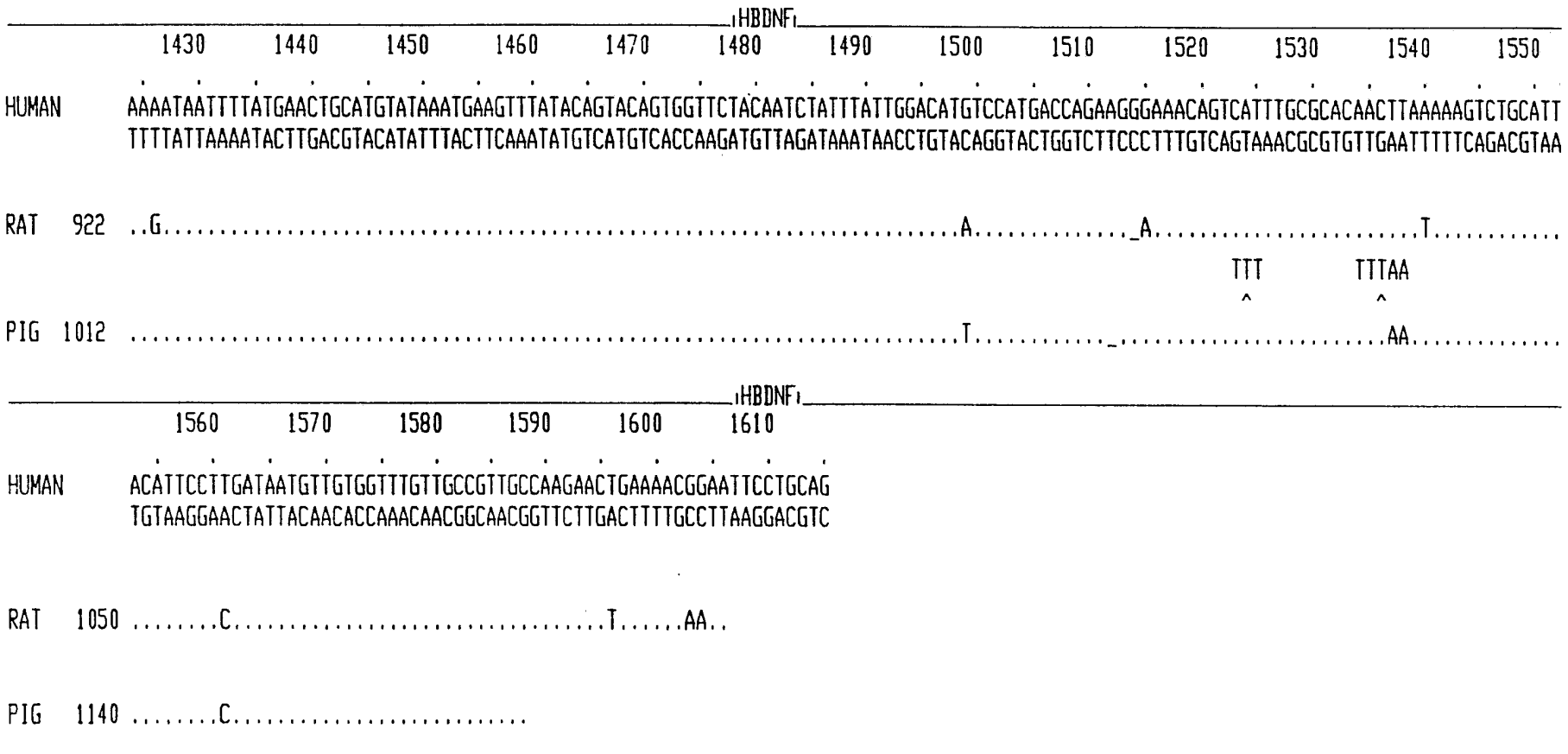


FIG. 3H

```
AGCGCATCGA GTGACTTTGG AGCTGGCCTT ATATTTGGAT CTCCCGGGCA GCTTTTTGGA
AACTCCTAGT GAAC ATG CTG TGC CTC AAG CCA GTG AAA TTA GGC TCC CTG
                Met Leu Cys Leu Lys Pro Val Lys Leu Gly Ser Leu
               -187                          -180

GAG GTG GGA CAC GGG CAG CAT GGT GGA GTT TTG GCC AGT GGT CGT GCA
Glu Val Gly His Gly Gln His Gly Gly Val Leu Ala Ser Gly Arg Ala
                    -170                                    -160

GTC CAA GGG GCT GGA TGG CAT GCT GGA CCC AAG CTC AGC TCA GCG TCC
Val Gln Gly Ala Gly Trp His Ala Gly Pro Lys Leu Ser Ser Ala Ser
                                  -150

GGA CCC AAT AAC AGT TTT ACC AAG GGA GCA GCT TTC TAT CCT GGC CAC
Gly Pro Asn Asn Ser Phe Thr Lys Gly Ala Ala Phe Tyr Pro Gly His
           -140                                    -130

ACT GAG GTG CAT AGC GTA ATG TCC ATG TTG TTC TAC ACT CTG ATC ACA
Thr Glu Val His Ser Val Met Ser Met Leu Phe Tyr Thr Leu Ile Thr
                          -120
```

FIG. 4

SUBSTITUTE SHEET

```
GCT TTT CTG ATC GGC ATA CAG GCG GAA CCA CAC TCA GAG AGC AAT GTC
Ala Phe Leu Ile Gly Ile Gln Ala Glu Pro His Ser Glu Ser Asn Val

   -110                                          -100

CCT GCA GGA CAC ACC ATC CCC CAA GTC CAC TGG ACT AAA CTT CAG CAT
Pro Ala Gly His Thr Ile Pro Gln Val His Trp Thr Lys Leu Gln His

                    -90                                      -80

TCC CTT GAC ACT GCC CTT CGC AGA GCC CGC AGC GCC CCG GCA GCG GCG
Ser Leu Asp Thr Ala Leu Arg Arg Ala Arg Ser Ala Pro Ala Ala Ala

                                  -70

ATA GCT GCA CGC GTG GCG GGG CAG ACC CGC AAC ATT ACT GTG GAC CCC
Ile Ala Ala Arg Val Ala Gly Gln Thr Arg Asn Ile Thr Val Asp Pro

          -60                                        -50

AGG CTG TTT AAA AAG CGG CGA CTC CGT TCA CCC CGT GTG CTG TTT AGC
Arg Leu Phe Lys Lys Arg Arg Leu Arg Ser Pro Arg Val Leu Phe Ser

                                -40
```

FIG. 4A

SUBSTITUTE SHEET

```
ACC CAG CCT CCC CGT GAA GCT GCA GAC ACT CAG GAT CTG GAC TTC GAG
Thr Gln Pro Pro Arg Glu Ala Ala Asp Thr Gln Asp Leu Asp Phe Glu

   -30                                                  -20

GTC GGT GGT GCT GCC CCC TTC AAC AGG ACT CAC AGG AGC AAG CGG TCA
Val Gly Gly Ala Ala Pro Phe Asn Arg Thr His Arg Ser Lys Arg Ser

                  -10                                          1

TCA TCC CAT CCC ATC TTC CAC AGG GGC GAA TTC TCG GTG TGT GAC AGT
Ser Ser His Pro Ile Phe His Arg Gly Glu Phe Ser Val Cys Asp Ser

                                    10

GTC AGC GTG TGG GTT GGG GAT AAG ACC ACC GCC ACA GAC ATC AAG GGC
Val Ser Val Trp Val Gly Asp Lys Thr Thr Ala Thr Asp Ile Lys Gly

        20                                              30

AAG GAG GTG ATG GTG TTG GGA GAG GTG AAC ATT AAC AAC AGT GTA TTC
Lys Glu Val Met Val Leu Gly Glu Val Asn Ile Asn Asn Ser Val Phe

                          40

AAA CAG TAC TTT TTT GAG ACC AAG TGC CGG GAC CCA AAT CCC GTT GAC
Lys Gln Tyr Phe Phe Glu Thr Lys Cys Arg Asp Pro Asn Pro Val Asp

50                                                  60
```

FIG. 4B

```
AGC GGG TGC CGG GGC ATT GAC TCA AAG CAC TGG AAC TCA TAT TGT ACC
Ser Gly Cys Arg Gly Ile Asp Ser Lys His Trp Asn Ser Tyr Cys Thr

                  70                                          80

ACG ACT CAC ACC TTT GTC AAG GCG CTG ACC GTG GAT GGC AAG CAG GCT
Thr Thr His Thr Phe Val Lys Ala Leu Thr Met Asp Gly Lys Gln Ala

                                    90

GCC TGG CGG TTT ATC CGG ATA GAT ACG GCC TGT GTG TGT GTG CTC AGC
Ala Trp Arg Phe Ile Arg Ile Asp Thr Ala Cys Val Cys Val Leu Ser

        100                                         110

AGG AAG GCT GTG AGA AGA GCC TGACCTGCCG ACACGCTCCC TCCCCCTGCC
Arg Lys Ala Val Arg Arg Ala

                        120

CCTTCTACAC TCTCCTGGGC CCCTCCCTAC CTCAACCTGT AAATTATTTT AAATTATAAG

GACTGCATGG TAATTTATAG TTTATACAGT TTTAAAGAAT CATTATTTAT TAAATTTTTG

GAAGCATCCT GTGTGCTGA
```

FIG. 4C

SUBSTITUTE SHEET

```
Sequence Range:  9 to 1142 | r/NT-3gene | R
Sequence Range: 33 to 1057 | h/NT-3gene | H

10          20          30          40          50
 *           *           *           *           *
GA TTCCATAA TGA CCC AGA CTC TTC CAG TCA GAT ATT AAC ACT TGT GTT      R
CT AAGGTATT ACT GGG TCT GAG AAG GTC AGT CTA TAA TTG TGA ACA CAA
            End Pro Arg Leu Phe Gln Ser Asp Ile Asn Thr Cys Val>

      40          50          60          70          80
       *           *           *           *           *
TGCCAGAA TAA CAC AGA CTC AGC TGC CAG AGC CTG CTC TTA ACA CCT GTG      H
ACGGTCTT ATT GTG TCT GAG TCG ACG GTC TCG GAC GAG AAT TGT GGA CAC
         End His Arg Leu Ser Cys Gln Ser Leu Leu Leu Thr Pro Val>

 60          70          80          90          100
  *           *           *           *           *
TCC TTC TTT CAG ATC TTA CAG GTG AAC AAG GTG ATG TCC ATC TTG TTT      R
AGG AAG AAA GTC TAG AAT GTC CAC TTG TTC CAC TAC AGG TAG AAC AAA
Ser Phe Phe Gln Ile Leu Gln Val Asn Lys Val

                                          Met Ser Ile Leu Phe>
                                          PREPRO___>>

        90          100          110          120          130
         *           *            *            *           *
TTT CCT TTT CAG ATC TTA CAG GTG AAC AAG GTG ATG TCC ATC TTG TTT      H
AAA GGA AAA GTC TAG AAT GTC CAC TTG TTC CAC TAC AGG TAG AAC AAA
Phe Pro Phe Gln Ile Leu Gln Val Asn Lys Val

                                          Met Ser Ile Leu Phe>
                                           PREPRO__>>
```

FIG. 5

```
   110         120         130         140         150
    *           *           *           *           *
TAT GTG ATA TTT CTT GCT TAT CTC CGT GGC ATC CAA GGC AAC AAC ATG      R
ATA CAC TAT AAA GAA CGA ATA GAG GCA CCG TAG GTT CCG TTG TTG TAC

Tyr Val Ile Phe Leu Ala Tyr Leu Arg Gly Ile Gln Gly Asn Asn Met>

          140         150         160         170
           *           *           *           *
TAT GTG ATA TTT CTC GCT TAT CTC CGT GGC ATC CAA GGT AAC AAC ATG      H
ATA CAC TAT AAA GAG CGA ATA GAG GCA CCG TAG GTT CCA TTG TTG TAC

Tyr Val Ile Phe Leu Ala Tyr Leu Arg Gly Ile Gln Gly Asn Asn Met>
```

```
      160         170         180         190         200
       *           *           *           *           *
GAT CAA AGG AGT TTG CCA GAA GAC TCT CTC AAT TCC CTC ATT ATC AAG      R
CTA GTT TCC TCA AAC GGT CTT CTG AGA GAG TTA AGG GAG TAA TAG TTC

Asp Gln Arg Ser Leu Pro Glu Asp Ser Leu Asn Ser Leu Ile Ile Lys>

180         190         200         210         220
 *           *           *           *           *
GAT CAA AGG AGT TTG CCA GAA GAC TCG CTC AAT TCC CTC ATT ATT AAG      H
CTA GTT TCC TCA AAC GGT CTT CTG AGC GAG TTA AGG GAG TAA TAA TTC

Asp Gln Arg Ser Leu Pro Glu Asp Ser Leu Asn Ser Leu Ile Ile Lys>
```

FIG. 5A

```
         210          220          230          240
          *            *            *            *
TTG ATC CAG GCG GAT ATC TTG AAA AAC AAG CTC TCC AAG CAG ATG GTA      R
AAC TAG GTC CGC CTA TAG AAC TTT TTG TTC GAG AGG TTC GTC TAC CAT

Leu Ile Gln Ala Asp Ile Leu Lys Asn Lys Leu Ser Lys Gln Met Val>

  230          240          250          260          270
   *            *            *            *            *
CTG ATC CAG GCA GAT ATT TTG AAA AAC AAG CTC TCC AAG CAG ATG GTG      H
GAC TAG GTC CGT CTA TAA AAC TTT TTG TTC GAG AGG TTC GTC TAC CAC

Leu Ile Gln Ala Asp Ile Leu Lys Asn Lys Leu Ser Lys Gln Met Val>

250          260          270          280          290
 *            *            *            *            *
 GAT GTT AAG GAA AAT TAC CAG AGC ACC CTG CCC AAA GCA GAG GCA CCC     R
 CTA CAA TTC CTT TTA ATG GTC TCG TGG GAC GGG TTT CGT CTC CGT GGG

 Asp Val Lys Glu Asn Tyr Gln Ser Thr Leu Pro Lys Ala Glu Ala Pro>

     280          290          300          310          320
      *            *            *            *            *
 GAC GTT AAG GAA AAT TAC CAG AGC ACC CTG CCC AAA GCT GAG GCT CCC     H
 CTG CAA TTC CTT TTA ATG GTC TCG TGG GAC GGG TTT CGA CTC CGA GGG

 Asp Val Lys Glu Asn Tyr Gln Ser Thr Leu Pro Lys Ala Glu Ala Pro>
```

FIG. 5B

SUBSTITUTE SHEET

```
300           310           320           330           340
 *             *             *             *             *
AGA GAA CCA GAG CAG GGA GAG GCC ACC AGG TCA GAA TTC CAG CCG ATG      R
TCT GTT GGT CTC GTC CCT CTC CGG TGG TCC AGT CTT AAG GTC GGC TAC

Arg Glu Pro Glu Gln Gly Gly Ala Thr Arg Ser Gly Phe Gln Pro Met

      330           340           350           360           370
        *             *             *             *             *
CGA GAG CCG GAG CAG GGA GGG CCC CGC AAG TCA GCA TTC CAG CCA GTG      H
GCT CTC GGC CTC GTC CCT CCC GGG GCG TTC AGT CGT AAG GTC GGT CAC

Arg Glu Pro Glu Gln Gly Gly Pro Arg Lys Ser Ala Phe Gln Pro Val>
```

```
   350           360           370           380           390
    *             *             *             *             *
ATT GCA ACA GAC ACA GAA CTA CTA CGG CAA CAG AGA CGC TAC AAT TCA      R
TAA CGT TGT CTG TGT CTT GAT GAT GCC GTT GTC TCT GCG ATG TTA AGT

Ile Ala Thr Asp Thr Glu Leu Leu Arg Gln Gln Arg Arg Tyr Asn Ser>

          380           390           400           410
            *             *             *             *
ATT GCA ATG GAC ACC GAA CTG CTG CGA CAA CAG AGA CGC TAC AAC TCA      H
TAA CGT TAC CTG TGG CTT GAC GAC GCT GTT GTC TCT GCG ATG TTG AGT

Ile Ala Met Asp Thr Glu Leu Leu Arg Gln Gln Arg Arg Tyr Asn Ser>
```

FIG. 5C

```
      400        410        420        430        440
       *          *          *          *          *
CCC CGG GTC CTG CTG AGT GAC AGC ACC CCT TTG GAG CCC CCT CCC TTA
GGG GCC CAG GAC GAC TCA CTG TCG TGG GGA AAC CTC GGG GGA GGG AAT

Pro Arg Val Leu Leu Ser Asp Ser Thr Pro Leu Glu Pro Pro Pro Leu>
```

R

```
420        430        440        450        460
 *          *          *          *          *
CCG CGG GTC CTG CTG AGC GAC ACG ACC CCC TTG GAG CCC CCG CCC TTG
GGC GCC CAG GAC GAC TCG CTG TGC TGG GGG AAC CTC GGG GGC GGG AAC

Pro Arg Val Leu Leu Ser Asp Thr Thr Pro Leu Glu Pro Pro Pro Leu>
```

H

```
      450        460        470        480
       *          *          *          *
TAT CTA ATG GAA GAT TAT GTG GGC AAC CCG GTG GTA ACC AAT AGA ACA
ATA GAT TAC CTT CTA ATA CAC CCG TTG GGC CAC CAT TGG TTA TCT TGT

Tyr Leu Met Glu Asp Tyr Val Gly Asn Pro Val Val Thr Asn Arg Thr>
```

R

```
470        480        490        500        510
 *          *          *          *          *
TAT CTC ATG GAG GAT TAC GTG GGC AGC CCC GTG GTG GCG AAC AGA ACA
ATA GAG TAC CTC CTA ATG CAC CCG TCG GGG CAC CAC CGC TTG TCT TGT

Tyr Leu Met Glu Asp Tyr Val Gly Ser Pro Val Val Ala Asn Arg Thr>
```

H

FIG. 5D

```
490          500          510          520          530
 *            *            *            *            *
 TCA CCA CGG AGG AAA CGC TAT GCA GAG CAT AAG AGT CAC CGA GGA GAG      R
 AGT GGT GCC TCC TTT GCG ATA CGT CTC GTA TTC TCA GTG GCT CCT CTC

 Ser Pro Arg Arg Lys Arg

                          Tyr Ala Glu His Lys Ser His Arg Gly Glu>
                            MATURE__>>

         520          530          540          550
           *            *            *            *
 TCA --- CGG CGG AAA CGG TAC GCG GAG CAT AAG AGT CAC CGA GGG GAG      H
 AGT --- GCC GCC TTT GCC ATG CGC CTC GTA TTC TCA GTG GCT CCC CTC

 Ser ___ Arg Arg Lys Arg

                          Tyr Ala Glu His Lys Ser His Arg Gly Glu>
                            MATURE__>>

 540          550          560          570          580
  *            *            *            *            *
 TAC TCA GTG TGT GAC AGT GAG AGC CTG TGG GTG ACC GAC AAG TCC TCA      R
 ATG AGT CAC ACA CTG TCA CTC TCG GAC ACC CAC TGG CTG TTC AGG AGT

 Tyr Ser Val Cys Asp Ser Glu Ser Leu Trp Val Thr Asp Lys Ser Ser>

560          570          580          590          600
 *            *            *            *            *
 TAC TCG GTA TGT GAC AGT GAG AGT CTG TGG GTG ACC GAC AAG TCA TCG      H
 ATG AGC CAT ACA CTG TCA CTC TCA GAC ACC CAC TGG CTG TTC AGT AGC

 Tyr Ser Val Cys Asp Ser Glu Ser Leu Trp Val Thr Asp Lys Ser Ser>
```

FIG. 5E

```
   590          600          610          620          630
    *            *            *            *            *
GCC ATT GAC ATT CGG GGA CAC CAG GTT ACA GTG TTG GGA GAG ATC AAA      R
CGG TAA CTG TAA GCC CCT GTG GTC CAA TGT CAC AAC CCT CTC TAG TTT

Ala Ile Asp Ile Arg Gly His Gln Val Thr Val Leu Gly Glu Ile Lys>

610          620          630          640          650
 *            *            *            *            *
GCC ATC GAC ATT CGG GGA CAC CAG GTC ACG GTG CTG GGG GAG ATC AAA      H
CGG TAG CTG TAA GCC CCT GTG GTC CAG TGC CAC GAC CCC CTC TAG TTT

Ala Ile Asp Ile Arg Gly His Gln Val Thr Val Leu Gly Glu Ile Lys>
```

```
      640          650          660          670          680
       *            *            *            *            *
ACC GGC AAC TCT CCT GTG AAA CAA TAT TTT TAT GAA ACG AGG TGT AAA      R
TGG CCG TTG AGA GGA CAC TTT GTT ATA AAA ATA CTT TGC TCC ACA TTT

Thr Gly Asn Ser Pro Val Lys Gln Tyr Phe Tyr Glu Thr Arg Cys Lys>

   660          670          680          690          700
    *            *            *            *            *
ACG GGC AAC TCT CCC GTC AAA CAA TAT TTT TAT GAA ACG CGA TGT AAG      H
TGC CCG TTG AGA GGG CAG TTT GTT ATA AAA ATA CTT TGC GCT ACA TTC

Thr Gly Asn Ser Pro Val Lys Gln Tyr Phe Tyr Glu Thr Arg Cys Lys>
                                50
```

FIG. 5F

```
         690           700           710           720
           *             *             *             *
GAA GCC AGG CCA GTC AAA AAC GGT TGC AGG GGG ATT GAT GAC AAA CAC       R
CTT CGG TCC GGT CAG TTT TTG CCA ACG TCC CCC TAA CTA CTG TTT GTG

Glu Ala Arg Pro Val Lys Asn Gly Cys Arg Gly Ile Asp Asp Lys His>

       710           720           730           740           750
         *             *             *             *             *
GAA GCC AGG CCG GTC AAA AAC GGT TGC AGG GGT ATT GAT GAT AAA CAC       H
CTT CGG TCC GGC CAG TTT TTG CCA ACG TCC CCA TAA CTA CTA TTT GTG

Glu Ala Arg Pro Val Lys Asn Gly Cys Arg Gly Ile Asp Asp Lys His>
```



```
730           740           750           760           770
  *             *             *             *             *
TGG AAC TCT CAG TGC AAA ACG TCG CAA ACC TAC GTC CCA GCA CTG ACT       R
ACC TTG AGA GTC ACG TTT TGC AGC GTT TGG ATG CAG GCT CGT GAC TGA

Trp Asn Ser Gln Cys Lys Thr Ser Gln Thr Tyr Val Arg Ala Leu Thr>

         760           770           780           790
           *             *             *             *
TGG AAC TCT CAG TGC AAA ACA TCC CAA ACC TAC GTC CGA GCA CTG ACT       H
ACC TTG AGA GTC ACG TTT TGT AGG GTT TGG ATG CAG GCT CGT CAC TGA

Trp Asn Ser Gln Cys Lys Thr Ser Gln Thr Tyr Val Arg Ala Leu Thr>
 75
```

FIG. 5G

```
780           790           800           810           820
  *             *             *             *             *
TCA GAA AAC AAC AAA CTC GTA GGC TGG CGC TGG ATA CGA ATA GAC ACT       R
AGT CTT TTG TTG TTT GAG CAT CCG ACC GCG ACC TAT GCT TAT CTG TGA

Ser Glu Asn Asn Lys Leu Val Gly Trp Arg Trp Ile Arg Ile Asp Thr>

800           810           820           830           840
  *             *             *             *             *
TCA GAG AAC AAT AAA CTC GTG GGC TGG CGG TGG ATA CGG ATA GAC ACG       H
AGT CTC TTG TTA TTT GAG CAC CCG ACC GCC ACC TAT GCC TAT CTG TGC

Ser Glu Asn Asn Lys Leu Val Gly Trp Arg Trp Ile Arg Ile Asp Thr>
                                        100

   830           840           850           860
     *             *             *             *
TCC TGT GTG TGT GCC TTG TCA AGA AAA ATC GGA AGA ACA TGA      R
AGG ACA CAC ACA CGG AAC AGT TCT TTT TAG CCT TCT TGT ACT

Ser Cys Val Cys Ala Leu Ser Arg Lys Ile Gly Arg Thr End>

850           860           870           880
  *             *             *             *
TCC TGT GTG TCT GCC TTG TCG AGA AAA ATC GGA AGA ACA TGA       H
AGG ACA CAC ACA CGG AAC AGC TCT TTT TAG CCT TCT TGT ACT

Ser Cys Val Cys Ala Leu Ser Arg Lys Ile Gly Arg Thr End>
                                                  119
```

FIG. 5H

```
870          880          890          900          910          920
*            *            *            *            *            *
ATTGGCATCT GTCCCCACAT ATAAATTATT ACTTTAAATT ATATGATATG CAT                R
TAACCGTAGA CAGGGGTGTA TATTTAATAA TGAAATTTAA TATACTATAC GTA

890          900          910          920          930          940
*            *            *            *            *            *
ATTGGCATCT CTCCCCATAT ATAAATTATT ACTTTAAATT ATATGATATG CAT                H
TAACCGTAGA GAGGGGTATA TATTTAATAA TGAAATTTAA TATACTATAC GTA

          930          940          950          960          970          980
          *            *            *            *            *            *
GTAGCATATA AATGTTTATA TTGTTTTTAT ATATTATAAG TTGACCTTTA TTTATTAAAC         R
CATCGTATAT TTACAAATAT AACAAAAATA TATAATATTC AACTGGAAAT AAATAATTTG

          950          960          970          980          990          1000
          *            *            *            *            *            *
GTAGCATATA AATGTTTATA TTGTTTTTAT ATAT-ATAAG TTGACCTTTA TTTATTAAAC         H
CATCGTATAT TTACAAATAT AACAAAAATA TATA-TATTC AACTGGAAAT AAATAATTTG
```

FIG. 51

```
        990       1000       1010       1020       1030       1040
          *          *          *          *          *          *
TTCAGCAACC CTTACAGTAT ATAAGCTTTT TTTTCTCAAT AAAATTCGTG TGCTTGCCTT   R
AAGTCGTTGG GAATGTCATA TATTCGAAAA AAAAGAGTTA TTTTAAGCAC ACGAACGGAA

     1010       1020       1030       1040       1050
        *          *          *          *          *
TTCAGCAACC CT-ACAGTAT ATAAGCTTAT CGATACCGTC GACCTCGAGG GGGGGCC/     H
AAGTCGTTGG GA-TGTCATA TATTCGAATA GCTATGGCAG CTGGAGCTCC CCCCCGG/

       1050       1060       1070       1080       1090       1100
          *          *          *          *          *          *
CGCTCAGGCC TCTCCCATCT GTTAACCTTG TTTTGTGATT GGGCTCTCGG GAACCTTCTG   R
GCGAGTCCGG AGAGGGTAGA CAATTGGAAC AAAACACTAA CCCGAGAGCC CTTGGAAGAC

       1110       1120       1130       1140
          *          *          *          *
TAAAACCTGT GTACACCAGT ATTTGGCATT CAGTATTGTC AA/                     R
ATTTTGGACA CATGTGGTCA TAAACCGTAA GTCATAACAG TT/
```

FIG. 5J

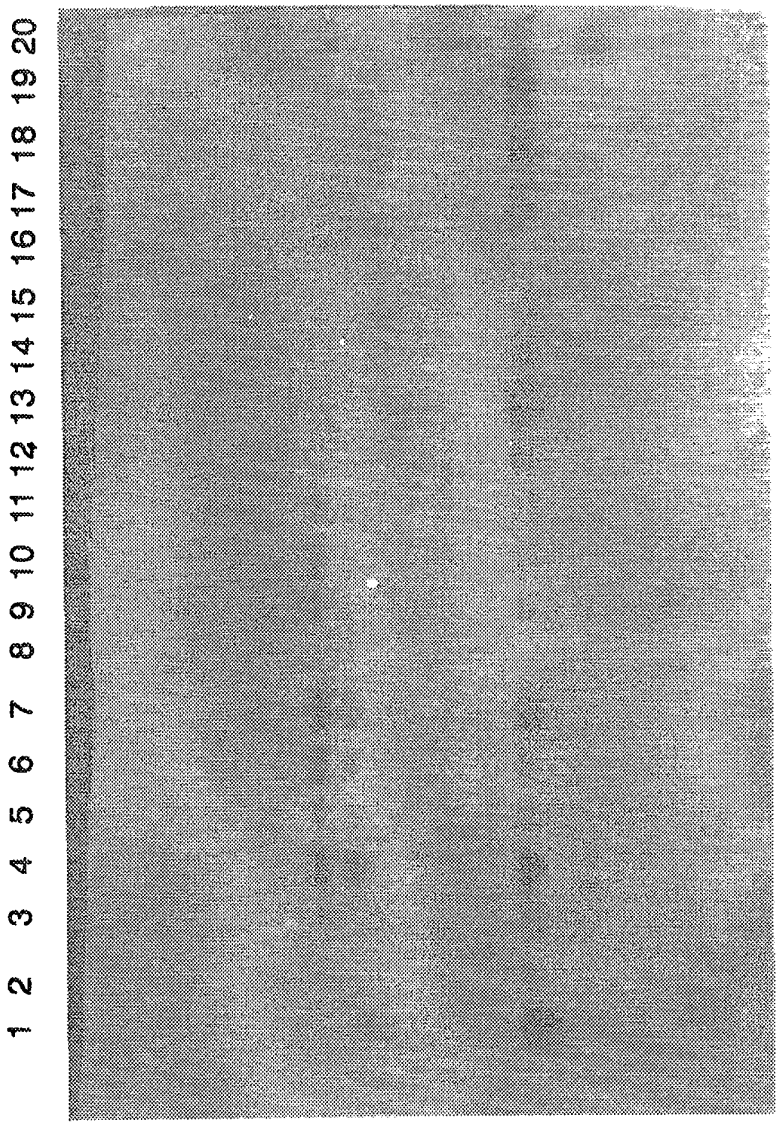
```
   5'GG GTC CTG CTG AGC GAC ACG ACC CCC TTG GAG CCC CCG
3'  CG  CC CAG GAC TCG CTG TGC TGG GGG AAC CTC GGG GGC

CCC TTG TAT CTC ATG GAG CAT TAC GTG GGC AGC CCC
GGG AAC ATA GAG TAC CTC CTA ATG CAC CCG TCG GGG

GTG GTG GCG AAC AGA ACA TCA CGG CGG AAA 3'
CAC CAC CGC TTG TCT TGT AGT GCC GCC TTT GC5'
```

FIG. 6

FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09792

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; C12N 15/18, 1/21, 15/67
US CL :530/399; 536/23.5; 435/69.1, 252.3, 320.1
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. : 530/399; 536/23.5; 435/69.1, 252.3, 320.1

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)

Dialog (Medline, Patents)
search terms: chimer, prepro, neurotroph

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. OF CELL BIOLOGY, Volume 108, No. 5, issued May 1989, T.J. Stoller et al., "The Propeptide of Preprosomatostatin Mediates Intracellular Transport and Secretion of alpha-Globin from Mammalian Cells", pages 1647-1656, see abstract.	1-50
Y	J. OF FERM AND BIOENG, Volume 68, No. 4, issued 1989, H. Oyama, et al., "Secretion of Escherichia coli Aminopeptidase P in Bacillus subtilis Using the Prepro-Structure Coding Region of Subtilisin Amylosacchariticus", pages 289-292, see abstract.	1-50
Y	PROC NATL ACAD SCI, USA, Volume 80, No 23, issued December 1983, S.D. Emr, "An MFalpha1-SUC2 (alpha-factor-invertase) gene fusion for study of protein localization and gene expression in yeast", pages 7080-7084, see abstract.	1-50
Y	DNA, Volume 7, No. 9, issued November 1988, B.R. Cullen, "Expression of a Cloned Human Interleukin-2 cDNA Is Enhanced by the Substitution of a Heterologous mRNA Leader Region", pages 645-650, see abstract.	1-50

[X] 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 part 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 11 February 1993	Date of mailing of the international search report 26 FEB 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer SHELLY GUEST CERMAK Telephone No. (703) 308-0196

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09792

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,923,808 (Matteucci) 08 May 1990, col. 1, line 55 - col. 2, line 14.	1-50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09792

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

I. Claims 1-15, drawn to a chimeric protein, classified in Class 530/399,

II. Claims 16-50, drawn to a DNA molecule, a vector, a host cell, and recombinant methods of making the protein, classified in Class 536/23.5 and Class 435/320.1, 69.1, 252.3.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims. (Telephone Practice)

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any addtional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention frist mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.