(54) Title: A MURINE SEVEN-TRANSMEMBRANE RECEPTOR, MUS MUSCULUS MHNEAA81

(57) Abstract: Mus musculus mHNEAA81 polypeptides and polynucleotides and method for producing such polypeptides by recombinant techniques are disclosed. Also disclosed are methods for screening for compounds which either agonize or antagonize Mus musculus mHNEAA81. Such compounds are expected to be useful in treatment of human diseases, including, but not limited to: infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson’s disease; acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial infarction; stroke; ulcers; asthma; allergies; benign prostatic hypertrophy; migraine; vomiting; psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, depression, delirium, dementia, and severe mental retardation; and dyskinesias, such as Huntington’s disease or Gilles de la Tourette’s syndrome.
A MURINE SEVEN-TRANSMEMBRANE RECEPTOR, MUS MUSCULUS MHNEAA81

Related Applications

This application claims priority of U.S. Provisional Application Serial No. 60/159,217, filed on October 13, 1999.

Field of the Invention

This invention relates to newly identified polypeptides and polynucleotides encoding such polypeptides, to their use in identifying compounds that may be agonists and/or antagonists that are potentially useful in therapy, and to production of such polypeptides and polynucleotides.

Background of the Invention

The drug discovery process is currently undergoing a fundamental revolution as it embraces 'functional genomics,’ that is, high throughput genome- or gene-based biology. This approach is rapidly superseding earlier approaches based on ‘positional cloning.' A phenotype, that is a biological function or genetic disease, would be identified and this would then be tracked back to the responsible gene, based on its genetic map position.

Functional genomics relies heavily on the various tools of bioinformatics to identify gene sequences of potential interest from the many molecular biology databases now available. There is a continuing need to identify and characterize further genes and their related polypeptides/proteins, as targets for drug discovery.

It is well established that many medically significant biological processes are mediated by proteins participating in signal transduction pathways that involve G-proteins and/or second messengers, e.g., cAMP (Lefkowitz, Nature, 1991, 351:353-354). Herein these proteins are referred to as proteins participating in pathways with G-proteins or PPG proteins. Some examples of these proteins include the G-protein coupled (GPC) receptors, such as those for adrenergic agents and dopamine (Kobilka, B.K., et al., Proc. Natl Acad. Sci., USA, 1987, 84:46-50; Kobilka, B.K., et al., Science, 1987, 238:650-656; Bunzow, J.R., et al., Nature, 1988, 336:783-787), G-proteins themselves, effector proteins, e.g.,

For example, in one form of signal transduction, the effect of hormone binding is activation of the enzyme, adenylate cyclase, inside the cell. Enzyme activation by hormones is dependent on the presence of the nucleotide, GTP. GTP also influences hormone binding. A G-protein connects the hormone receptor to adenylate cyclase. G-protein was shown to exchange GTP for bound GDP when activated by a hormone receptor. The GTP-carrying form then binds to activated adenylate cyclase. Hydrolysis of GTP to GDP, catalyzed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G-protein serves a dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the duration of the signal.

The membrane protein gene superfamily of G-protein coupled receptors has been characterized as having seven putative transmembrane domains. The domains are believed to represent transmembrane α-helices connected by extracellular or cytoplasmic loops. G-protein coupled receptors include a wide range of biologically active receptors, such as hormone, viral, growth factor and neuroreceptors.

G-protein coupled receptors (otherwise known as 7TM receptors) have been characterized as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. The G-protein family of coupled receptors includes dopamine receptors which bind to neuroleptic drugs used for treating psychotic and neurological disorders. Other examples of members of this family include, but are not limited to: calcitonin, adrenergic, endothelin, cAMP, adenosine, muscarinic, acetylcholine, serotonin, histamine, thrombin, kinin, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorant, and cytomegalovirus receptors.

Most G-protein coupled receptors have single conserved cysteine residues in each of the first two extracellular loops which form disulfide bonds that are believed to stabilize functional protein structure. The 7 transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 has been implicated in signal transduction.
Phosphorylation and lipidation (palmitylation or farnesylation) of cysteine residues can influence signal transduction of some G-protein coupled receptors. Most G-protein coupled receptors contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several G-protein coupled receptors, such as the β-adrenoreceptor, phosphorylation by protein kinase A and/or specific receptor kinases mediates receptor desensitization.

For some receptors, the ligand binding sites of G-protein coupled receptors are believed to comprise hydrophilic sockets formed by several G-protein coupled receptor transmembrane domains, said sockets being surrounded by hydrophobic residues of the G-protein coupled receptors. The hydrophilic side of each G-protein coupled receptor transmembrane helix is postulated to face inward and form a polar ligand binding site. TM3 has been implicated in several G-protein coupled receptors as having a ligand binding site, such as the TM3 aspartate residue. TM5 serines, a TM6 asparagine and TM6 or TM7 phenylalanines or tyrosines are also implicated in ligand binding.

G-protein coupled receptors can be intracellularly coupled by heterotrimeric G-proteins to various intracellular enzymes, ion channels and transporters (see, Johnson et al., Endoc. Rev., 1989, 10:317-331). Different G-protein α-subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of G-protein coupled receptors has been identified as an important mechanism for the regulation of G-protein coupling of some G-protein coupled receptors. G-protein coupled receptors are found in numerous sites within a mammalian host.

Over the past 15 years, nearly 350 therapeutic agents targeting 7 transmembrane (7 TM) receptors have been successfully introduced onto the market.

Summary of the Invention

The present invention relates to Mus musculus mHNEAA81, in particular Mus musculus mHNEAA81 polypeptides and Mus musculus mHNEAA81 polynucleotides, recombinant materials and methods for their production. In another aspect, the invention relates to methods for identifying agonists and antagonists/inhibitors of the Mus musculus mHNEAA81 gene. This invention further relates to the generation of in vitro and in vivo comparison data relating to the polynucleotides and polypeptides in order to predict oral
absorption and pharmacokinetics in man of compounds that either agonize or antagonize the biological activity of such polynucleotides or polypeptides. Such a comparison of data will enable the selection of drugs with optimal pharmacokinetics in man, i.e., good oral bioavailability, blood-brain barrier penetration, plasma half-life, and minimum drug interaction.

The present invention further relates to methods for creating transgenic animals, which over-express or under-express or have regulatable expression of a mHNEAA81 gene and "knock-out" animals, preferably mice, in which an animal no longer expresses a mHNEAA81 gene. Furthermore, this invention relates to transgenic and knock-out animals obtained by using these methods. Such animal models are expected to provide valuable insight into the potential pharmacological and toxicological effects in humans of compounds that are discovered by the aforementioned screening methods as well as other methods. An understanding of how a Mus musculus mHNEAA81 gene functions in these animal models is expected to provide an insight into treating and preventing human diseases including, but not limited to: infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial infarction; stroke; ulcers; asthma; allergies; benign prostatic hypertrophy; migraine; vomiting; psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, depression, delirium, dementia, and severe mental retardation; and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome, hereinafter referred to as "the Diseases", amongst others.

In still another aspect, the invention relates to methods to identify agonists and antagonists using the materials provided by the invention. In particular, the preferred method for identifying agonist or antagonist of mHNEAA81 receptor of the present invention comprises:

contacting a cell expressing on the surface thereof the receptor, said receptor being associated with a second component capable of providing a detectable signal in response to the
binding of a compound to said receptor, with a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound binds to and activates or inhibits the receptor by measuring the level of a signal generated from the interaction of the compound with the receptor.

In a further preferred embodiment, the method further comprises conducting the identification of agonist or antagonist in the presence of labeled or unlabeled di-adenosine tetraphosphate (hereinafter referred to as "AP4A"), di-adenosine hexaphosphate (hereinafter referred to as "AP6A"), di-adenosine pentaphosphate (hereinafter referred to as "AP5A"), or deoxy-uridine di-phosphate (hereinafter referred to as "d-UDP").

In another embodiment of the method for identifying agonist or antagonist of a mHNEAA81 receptor of the present invention comprises:

determining the inhibition of binding of a ligand to cells which have the receptor on the surface thereof, or to cell membranes containing the receptor, in the presence of a candidate compound under conditions to permit binding to the receptor, and determining the amount of ligand bound to the receptor, such that a compound capable of causing reduction of binding of a ligand is an agonist or antagonist. Preferably, the ligand is AP4A, AP5A, AP6A, or d-UDP. Yet more preferably, AP4A, AP5A, AP6A or d-UDP is labeled.

Description of the Invention

In a first aspect, the present invention relates to Mus musculus mHNEAA81 polypeptides. Such polypeptides include isolated polypeptides comprising an amino acid sequence having at least a 95% identity, most preferably at least a 97-99% identity, to that of SEQ ID NO:2 over the entire length of SEQ ID NO:2. Such polypeptides include:

(a) an isolated polypeptide comprising the amino acid of SEQ ID NO:2;

(b) an isolated polypeptide encoded by a polynucleotide comprising the sequence contained in SEQ ID NO:1;

(c) an isolated polypeptide comprising a polypeptide sequence having at least a 95%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
(d) an isolated polypeptide having at least a 95%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;

(e) the polypeptide sequence of SEQ ID NO:2; and

(f) variants and fragments thereof; and portions of such polypeptides in (a) to (e) that generally contain at least 30 amino acids, more preferably at least 50 amino acids, thereof.

Polypeptides of the present invention are members of the 7 Transmembrane Receptor (G-Protein Coupled Receptor) family of polypeptides. They are, therefore, of interest, because understanding the biological activities of mHNEAA81 in murine would help to understand the biological function of its human counterpart, human HNEAA81, and other related genes. In addition, G-protein coupled receptors, more than any other gene family, are the objects of pharmaceutical intervention. Furthermore, the polypeptides of the present invention can be used to establish assays to predict oral absorption and pharmacokinetics in man and thus enhance compound and formulation design, among others. These properties, either alone or in the aggregate, are hereinafter referred to as "Mus musculus mHNEAA81 activity" or "Mus musculus mHNEAA81 polypeptide activity" or “biological activity of mHNEAA81.” Preferably, a polypeptide of the present invention exhibits at least one biological activity of Mus musculus mHNEAA81.

Polypeptides of the present invention also include variants of the aforementioned polypeptides, including alleles and splice variants. Such polypeptides vary from the reference polypeptide by insertions, deletions, and substitutions that may be conservative or non-conservative. Particularly preferred variants are those in which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3, from 3 to 2, from 2 to 1 or 1 amino acids are inserted, substituted, or deleted, in any combination. Particularly preferred primers will have between 20 and 25 nucleotides.

Preferred fragments of polypeptides of the present invention include an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids from the amino acid sequence of SEQ ID NO: 2, or an isolated polypeptide comprising an amino acid sequence having at least 15, 20, 30, 40, 50 or 100 contiguous amino acids truncated or deleted from the amino acid sequence of SEQ ID NO: 2.
Also preferred are biologically active fragments that mediate activities of mHNEAA81, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those fragments that are antigenic or immunogenic in an animal, especially in a human. Particularly preferred are fragments comprising receptors or domains of enzymes that confer a function essential for viability of Mus musculus or the ability to initiate, or maintain cause the Diseases in an individual, particularly a human.

Fragments of the polypeptides of the invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, these variants may be employed as intermediates for producing the full-length polypeptides of the invention.

The polypeptides of the present invention may be in the form of a “mature” protein or may be a part of a larger protein such as a fusion protein. It is often advantageous to include an additional amino acid sequence that contains secretory or leader sequences, pro-sequences, sequences that aid in purification, for instance, multiple histidine residues, or an additional sequence for stability during recombinant production.

The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the refers by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination.

Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

In a further aspect, the present invention relates to Mus musculus mHNEAA81 polynucleotides. Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide having at least a 95% identity, to the amino
acid sequence of SEQ ID NO: 2, over the entire length of SEQ ID NO: 2. In this regard, polypeptides which have at least a 97% identity are highly preferred, while those with at least a 98-99% identity are more highly preferred, and those with at least a 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the nucleotide sequence contained in SEQ ID NO: 1 encoding the polypeptide of SEQ ID NO: 2.

Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence having at least a 95% identity, to a nucleotide sequence encoding a polypeptide of SEQ ID NO: 2, over the entire coding region. In this regard, polynucleotides which have at least a 97% identity are highly preferred, while those with at least a 98-99% identity are more highly preferred, and those with at least a 99% identity are most highly preferred.

Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence having at least a 95% identity, to SEQ ID NO: 1 over the entire length of SEQ ID NO: 1. In this regard, polynucleotides which have at least a 97% identity are highly preferred, while those with at least a 98-99% identity are more highly preferred, and those with at least a 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the polynucleotide of SEQ ID NO: 1, as well as the polynucleotide of SEQ ID NO: 1.

The invention also provides polynucleotides that are complementary to all the above-described polynucleotides.

The nucleotide sequence of SEQ ID NO: 1 shows homology with qa44f10.s1 Soares_NhHMPu_S1 Homo sapiens cDNA clone (NCI-CGAP http://www.ncbi.nlm.nih.gov/ncicgap. National Cancer Institute, Cancer Genome Anatomy Project (CGAP Unpublished 1997)). The nucleotide sequence of SEQ ID NO: 1 is a cDNA sequence and comprises a polypeptide encoding sequence (1 to 1014) encoding a polypeptide of 337 amino acids, the polypeptide of SEQ ID NO: 2. The nucleotide sequence encoding the polypeptide of SEQ ID NO: 2 may be identical to the polypeptide encoding sequence of SEQ ID NO: 1 or it may be a sequence other than SEQ ID NO: 1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the
polypeptide of SEQ ID NO: 2. The polypeptide of the SEQ ID NO: 2 is structurally related to other proteins of the 7 Transmembrane Receptor family, having homology and/or structural similarity with Human KIAA0001 (Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K. and Tabata, S. DNA Res. 1 (1), 27-35 (1994). Preferred polypeptides and polynucleotides of the present invention are expected to have, inter alia, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, preferred polypeptides and polynucleotides of the present invention have at least one mHNEAA81 activity.


When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself; or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro-protein sequence, or other fusion peptide portions. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz, et al., Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also comprise non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further embodiments of the present invention include polynucleotides encoding polypeptide variants that comprise the amino acid sequence of SEQ ID NO: 2 and in which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3,
from 3 to 2, from 1 to 1 or 1 amino acid residues are substituted, deleted or added, in any combination. Particularly preferred probes will have between 30 and 50 nucleotides, but may have between 100 and 200 contiguous nucleotides of the polynucleotide of SEQ ID NO: 1.

A preferred embodiment of the invention is a polynucleotide consisting of or comprising nucleotide 1 to the nucleotide immediately upstream of or including nucleotide 1011 set forth in SEQ ID NO: 1, both of which encode a mHNEAA81 polypeptide.

The invention also includes a polynucleotide consisting of or comprising a polynucleotide of the formula:

\[ X-(R1)m-(R2)-(R3)n-Y \]

wherein, at the 5' end of the molecule, X is hydrogen, a metal or a modified nucleotide residue, or together with Y defines a covalent bond, and at the 3' end of the molecule, Y is hydrogen, a metal, or a modified nucleotide residue, or together with X defines the covalent bond, each occurrence of R1 and R3 is independently any nucleic acid residue or modified nucleic acid residue, m is an integer between 1 and 3000 or zero, n is an integer between 1 and 3000 or zero, and R2 is a nucleic acid sequence or modified nucleic acid sequence of the invention, particularly the nucleic acid sequence set forth in SEQ ID NO:1 or a modified nucleic acid sequence thereof. In the polynucleotide formula above, R2 is oriented so that its 5' end nucleic acid residue is at the left, bound to R1, and its 3' end nucleic acid residue is at the right, bound to R3. Any stretch of nucleic acid residues denoted by either R1 and/or R2, where m and/or n is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer. Where, in a preferred embodiment, X and Y together define a covalent bond, the polynucleotide of the above formula is a closed, circular polynucleotide, which can be a double-stranded polynucleotide wherein the formula shows a first strand to which the second strand is complementary. In another preferred embodiment m and/or n is an integer between 1 and 1000. Other preferred embodiments of the invention are provided where m is an integer between 1 and 50, 100 or 500, and n is an integer between 1 and 50, 100, or 500.

Polynucleotides that are identical, or are substantially identical to a nucleotide sequence of SEQ ID NO: 1, may be used as hybridization probes for cDNA and genomic
DNA or as primers for a nucleic acid amplification (PCR) reaction, to isolate full-length cDNAs and genomic clones encoding polypeptides of the present invention and to isolate cDNA and genomic clones of other genes (including genes encoding homologs and orthologs from species other than Mus musculus) that have a high sequence identity to SEQ ID NO: 1. Typically these nucleotide sequences are 95% identical to that of the referent. Preferred probes or primers will generally comprise at least 15 nucleotides, preferably, at least 30 nucleotides and may have at least 50 nucleotides, and may even have at least 100 nucleotides. Particularly preferred primers will have between 20 and 25 nucleotides.

A polynucleotide encoding a polypeptide of the present invention, including homologs and orthologs from a species other than Mus musculus, may be obtained by a process comprising the steps of screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof, preferably of at least 15 nucleotides in length; and isolating full-length cDNA and genomic clones comprising said polynucleotide sequence. Such hybridization techniques are well known to the skilled artisan. Preferred stringent hybridization conditions include overnight incubation at 42oC in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1x SSC at about 65oC. Thus, the present invention also includes isolated polynucleotides, preferably of at least 100 nucleotides in length, obtained by screening an appropriate library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof, preferably of at least 15 nucleotides.

The skilled artisan will appreciate that, in many cases, an isolated cDNA sequence will be incomplete, in that the region coding for the polypeptide is cut short at the 5' end of the cDNA. This is a consequence of reverse transcriptase, an enzyme with inherently low 'processivity' (a measure of the ability of the enzyme to remain attached to the template during the polymerization reaction), failing to complete a DNA copy of the mRNA template during 1st strand cDNA synthesis.
There are several methods available and well known to those skilled in the art to obtain full-length cDNAs, or extend short cDNAs, for example, those based on the method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman, et al., Proc. Natl. Acad. Sci., USA 85, 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon™ technology (Clontech Laboratories Inc.), for example, have significantly simplified the search for longer cDNAs. In the Marathon™ technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adaptor' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the 'missing' 5' end of the cDNA using a combination of gene specific and adaptor specific oligonucleotide primers. The PCR reaction is then repeated using 'nested' primers, that is, primers designed to anneal within the amplified product (typically an adaptor specific primer that anneals further 3' in the adaptor sequence and a gene specific primer that anneals further 5' in the known gene sequence). The products of this reaction can then be analyzed by DNA sequencing and a full-length cDNA constructed either by joining the product directly to the existing cDNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression systems and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis, et al., Basic Methods in Molecular Biology (1986) and Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Preferred methods of introducing polynucleotides into host cells include, for instance, calcium
phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, E.coli, Streptomyces and Bacillus subtilis cells; fungal cells, such as yeast cells and Aspergillus cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may comprise control regions that regulate as well as engender expression. Generally, any system or vector that is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook, et al., MOLECULAR CLONING, A LABORATORY MANUAL (supra).

If a polypeptide of the present invention is to be expressed for use in screening assays, it is generally preferred that the polypeptide be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If the polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide. If produced intracellularly, the cells must first be lysed before the polypeptide is recovered.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose
chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well-known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

The polynucleotide sequences of the present invention are also valuable for chromosome localization studies. The polynucleotide sequence, or fragment(s) thereof, is specifically targeted to, and can hybridize with, a particular location on an individual human chromosome. The mapping of these sequences to human chromosomes according to the present invention is an important first step in correlating homologous human polynucleotide sequences with gene associated disease in humans.

Precise chromosomal localizations for a polynucleotide sequence (gene fragment, etc.) can be determined using Radiation Hybrid (RH) Mapping (Walter, M., et al. (1994) Nature Genetics 7, 22-28), for example. A number of RH panels are available, including mouse, rat, baboon, zebrafish and human. RH mapping panels are available from a number of sources, for example Research Genetics (Huntsville, AL, USA). To determine the chromosomal location of a polynucleotide sequence using these panels, PCR reactions are performed using primers, designed to the polynucleotide sequence of interest, on the RH DNAs of the panel. Each of these DNAs contains random genomic fragments from the species of interest. These PCR reactions result in a number of scores, one for each RH DNA in the panel, indicating the presence or absence of the PCR product of the polynucleotide sequence of interest. These scores are compared with scores created using PCR products from genomic sequences of known location, usually using an on-line resource such as that available at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, USA website (http://www.genome.wi.mit.edu/). Once a polynucleotide sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data for that species. Also, as a consequence of synteny, where knowledge of the position of a gene on a chromosome of one species can be used to determine the likely position of the orthologous gene on the chromosome of another species, this knowledge can then be used to identify candidate genes for human disease. Thus the localization of a polynucleotide sequence of interest to a
specific mouse chromosomal location can be used to predict the localization of the
orthologous human gene on the corresponding human chromosome. From this data,
potential disease association may be inferred from genetic map sources such as, for
example, V. McKusick, Mendelian Inheritance in Man (available on-line through Johns
Hopkins University Welch Medical Library). The relationship between genes and diseases
that have been mapped to the same chromosomal region are then identified through linkage
analysis (co-inheritance of physically adjacent genes).

Mus musculus mHNEAA81 gene products can be expressed in transgenic animals.
Animals of any species, including, but not limited to: mice, rats, rabbits, guinea pigs, dogs,
cats, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys,
chimpanzees, may be used to generate mHNEAA81 transgenic animals.

This invention further relates to a method of producing transgenic animals,
preferably Mus musculus, over-expressing mHNEAA81, which method may comprise the
introduction of several copies of a segment comprising at least the polynucleotide sequence
encoding SEQ ID NO: 2 with a suitable promoter into the cells of a Mus musculus embryo,
or the cells of another species, at an early stage.

This invention further relates to a method of producing transgenic animals,
preferably Mus musculus, under-expressing or regulatably expressing mHNEAA81, which
method may comprise the introduction of a weak promoter or a regulatable promoter (e.g.,
an inducible or repressible promoter) respectively, expressibly linked to the polynucleotide
sequence of SEQ ID NO:1 into the cells of a Mus musculus embryo at an early stage.

This invention also relates to transgenic animals, characterized in that they are
obtained by a method, as defined above.

Any technique known in the art may be used to introduce a Mus musculus
mHNEAA81 transgene into animals to produce a founder line of animals. Such techniques
include, but are not limited to: pronuclear microinjection (U.S. Patent No. 4,873,191);
retrovirus mediated gene transfer into germ lines (Van der Putten, et al., Proc. Natl. Acad.
Sci., USA 82: 6148-6152 (1985); gene targeting in embryonic stem cells (Thompson, et al.,

A further aspect of the present invention involves gene targeting by homologous recombination in embryonic stem cells to produce a transgenic animal with a mutation in a mHNEAA81 gene ("knock-out" mutation). In such so-called "knock-out" animals, there is inactivation of the mHNEAA81 gene or altered gene expression, such that the animals are useful to study the function of the mHNEAA81 gene, thus providing animals models of human disease, which are otherwise not readily available through spontaneous, chemical or irradiation mutagenesis. Another aspect of the present invention involves the generation of so-called "knock-in" animals in which a portion of a wild-type gene is fused to the cDNA of a heterologous gene.

This invention further relates to a method of producing "knock-out" animals, preferably mice, no longer expressing mHNEAA81. By using standard cloning techniques, a Mus musculus mHNEAA81 cDNA (SEQ ID NO: 1) can be used as a probe to screen suitable libraries to obtain the murine mHNEAA81 genomic DNA clone. Using the murine genomic clone, the method used to create a knockout mouse is characterized in that:

a suitable mutation is produced in the polynucleotide sequence of the murine mHNEAA81 genomic clone, which inhibits the expression of a gene encoding murine mHNEAA81, or inhibits the activity of the gene product;

said modified murine mHNEAA81 polynucleotide is introduced into a homologous segment of murine genomic DNA, combined with an appropriate marker, so as to obtain a labeled sequence comprising said modified murine genomic DNA;

said modified murine genomic DNA comprising the modified polynucleotide is transfected into embryonic stem cells and correctly targeted events selected in vitro; then

said stem cells are re-injected into a mouse embryo; then

said embryo is implanted into a female recipient and brought to term as a chimera which transmits said mutation through the germline; and

homozygous recombinant mice are obtained at the F2 generation which are recognizable by the presence of the marker.
Various methods for producing mutations in non-human animals are contemplated and well known in the art. In a preferred method, a mutation is generated in a murine mHNEAA81 allele by the introduction of a DNA construct comprising DNA of a gene encoding murine mHNEAA81, which murine gene contains the mutation. The mutation is targeted to the allele by way of the DNA construct. The DNA of the gene encoding murine mHNEAA81 comprised in the construct may be foreign to the species of which the recipient is a member, may be native to the species and foreign only to the individual recipient, may be a construct comprised of synthetic or natural genetic components, or a mixture of these. The mutation may constitute an insertion, deletion, substitution, or combination thereof. The DNA construct can be introduced into cells by, for example, calcium-phosphate DNA co-precipitation. It is preferred that a mutation be introduced into cells using electroporation, microinjection, virus infection, ligand-DNA conjugation, virus-ligand-DNA conjugation, or liposomes.

Another embodiment of the instant invention relates to "knock-out" animals, preferably mice, obtained by a method of producing recombinant mice as defined above, among others.

Another aspect of this invention provides for in vitro mHNEAA81 "knock-outs", i.e., tissue cultures. Animals of any species, including, but not limited to: mice, rats, rabbits, guinea pigs, dogs, cats, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, chimpanzees, may be used to generate in vitro mHNEAA81 "knock-outs". Methods for "knocking out" genes in vitro are described in Galli-Taliadoros, et al., Journal of Immunological Methods 181: 1-15 (1995).

Transgenic, "knock-in", and "knock-out" animals, as defined above, are a particularly advantageous model, from a physiological point of view, for studying Transmembrane Receptors. Such animals will be valuable tools to study the functions of a mHNEAA81 gene. Moreover, such animal models are expected to provide information about potential toxicological effects in humans of any compounds discovered by an aforementioned screening method, among others. An understanding of how a Mus musculus mHNEAA81 gene functions in these animal models is expected to provide an insight into treating and preventing human diseases including, but not limited to: infections
such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial infarction; stroke; ulcers; asthma; allergies; benign prostatic hypertrophy; migraine; vomiting; psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, depression, delirium, dementia, and severe mental retardation; and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

Polypeptides of the present invention are responsible for many biological functions, including many disease states, in particular the Diseases mentioned herein. It is, therefore, an aspect of the invention to devise screening methods to identify compounds that stimulate (agonists) or that inhibit (antagonists) the function of the polypeptide, such as agonists, antagonists and inhibitors. Accordingly, in a further aspect, the present invention provides for a method of screening compounds to identify those that stimulate or inhibit the function of the polypeptide. In general, agonists or antagonists may be employed for therapeutic and prophylactic purposes for the Diseases mentioned herein mentioned. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such agonists and antagonists so identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide; or may be structural or functional mimetics thereof (see Coligan, et al., Current Protocols in Immunology 1(2): Chapter 5 (1991)).

The screening method may simply measure the binding of a candidate compound to the polypeptide, or to cells or membranes bearing the polypeptide, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, a screening method may involve measuring or, qualitatively or quantitatively, detecting the competition of binding of a candidate compound to the polypeptide with a labeled competitor (e.g., agonist or antagonist). Further, screening methods may test whether the candidate compound results in a signal generated by an agonist or antagonist of the polypeptide, using detection systems appropriate to cells bearing the polypeptide. Antagonists are generally assayed in the presence of a known agonist and an effect on activation by the agonist by the presence of the candidate compound is observed. Further, screening methods may simply comprise the steps of mixing a candidate compound with a
solution comprising a polypeptide of the present invention, to form a mixture, measuring Mus musculus mHNEAA81 activity in the mixture, and comparing a Mus musculus mHNEAA81 activity of the mixture to a control mixture which contains no candidate compound.

Polypeptides of the present invention may be employed in conventional low capacity screening methods and also in high-throughput screening (HTS) formats. Such HTS formats include not only the well-established use of 96- and, more recently, 384-well microtiter plates but also emerging methods such as the nanowell method described by Schullek, et al., Anal Biochem., 246, 20-29, (1997).

Fusion proteins, such as those made from Fc portion and Mus musculus mHNEAA81 polypeptide, as herein described, can also be used for high-throughput screening assays to identify antagonists of antagonists of the polypeptide of the present invention (see D. Bennett, et al., J. Mol. Recognition, 8:52-58 (1995); and K. Johanson, et al., J. Biol. Chem., 270(16): 9459-9471 (1995)).

One screening technique includes the use of cells which express the mHNEAA81 receptor polypeptide (for example, transfected CHO cells) in a system which measures extracellular pH or intracellular calcium changes caused by receptor activation. In this technique, compounds may be contacted with cells expressing the receptor polypeptide of the present invention. A second messenger response, e.g., signal transduction, pH changes, or changes in calcium level, is then measured to determine whether the potential compound activates or inhibits the receptor.

Another screening technique involves expressing the mHNEAA81 polypeptide in which the receptor is linked to phospholipase C or D. Representative examples of such cells include, but are not limited to, endothelial cells, smooth muscle cells, and embryonic kidney cells. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase second signal.

Another method involves screening for compounds which are antagonists, and thus inhibit activation of the mHNEAA81 receptor polypeptide by determining inhibition of binding of labeled ligand, such as AP4A, AP5A, AP6A, or d-UDP, to cells which have the
receptor on the surface thereof, or cell membranes containing the receptor. Such a method involves transfecting an eukaryotic cell with DNA encoding the mHNEAA81 polypeptide such that the cell expresses the receptor on its surface. The cell is then contacted with a potential antagonist in the presence of a labeled form of a ligand, such as AP4A, AP5A, AP6A, or d-UDP. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity associated with transfected cells or membrane from these cells. If the compound binds to the receptor, the binding of labeled ligand to the receptor is inhibited as determined by a reduction of labeled ligand which binds to the receptors. This method is called binding assay. Naturally, this same technique can be used to look for an agonist.

Another method involves screening for receptor inhibitors by determining inhibition or stimulation of receptor-mediated cAMP and/or adenylate cyclase accumulation. Such a method involves transfecting a eukaryotic cell with the mHNEAA81 polypeptide receptor to express the receptor on the cell surface. The cell is then exposed to potential antagonists, in the presence of the mHNEAA81 polypeptide ligand, such as AP4A, AP5A, AP6A, or d-UDP. The changes in levels of cAMP is then measured over a defined period of time, for example, by radio-immuno or protein binding assays (for example using Flashplates or a scintillation proximity assay). Changes in cAMP levels can also be determined by directly measuring the activity of the enzyme, adenyllyl cyclase, in broken cell preparations. If the potential antagonist binds the receptor, and thus inhibits mHNEAA81 polypeptide-ligand binding, the levels of mHNEAA81 polypeptide-mediated cAMP, or adenylate cyclase activity, will be reduced or increased.

Another screening procedure involves the use of mammalian cells (CHO, HEK 293, Xenopus Oocytes, RBL-2H3, etc.) which are transfected to express the receptor of interest. The cells are loaded with an indicator dye that produces a fluorescent signal when bound to calcium, and the cells are contacted with a test substance and a receptor agonist, such as AP4A, AP5A, AP6A, or d-UDP. Any change in fluorescent signal is measured over a defined period of time using, for example, a fluorescence spectrophotometer or a fluorescence imaging plate reader. A change in the fluorescence signal pattern generated by the ligand indicates that a compound is a potential antagonist or agonist for the receptor.
Another screening procedure involves use of mammalian cells (CHO, HEK293, Xenopus Oocytes, RBL-2H3, etc.) which are transfected to express the receptor of interest, and which are also transfected with a reporter gene construct that is coupled to activation of the receptor (for example, luciferase or beta-galactosidase behind an appropriate promoter). The cells are contacted with a test substance and the receptor agonist (ligand), such as AP4A, AP5A, AP6A, or d-UDP, and the signal produced by the reporter gene is measured after a defined period of time. The signal can be measured using a luminometer, spectrophotometer, fluorometer, or other such instrument appropriate for the specific reporter construct used. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor.

Another screening technique for antagonists or agonists involves introducing RNA encoding the mHNEAA81 polypeptide into Xenopus oocytes (or CHO, HEK 293, RBL-2H3, etc.) to transiently or stably express the receptor. The receptor oocytes are then contacted with the receptor ligand, such as AP4A, AP5A, AP6A, or d-UDP, and a compound to be screened. Inhibition or activation of the receptor is then determined by detection of a signal, such as, cAMP, calcium, proton, or other ions.

Another screening method for agonists and antagonists relies on the endogenous pheromone response pathway in the yeast, Saccharomyces cerevisiae. Heterothallic strains of yeast can exist in two mitotically stable haploid mating types, MATa and MATa. Each cell type secretes a small peptide hormone that binds to a G-protein coupled receptor on opposite mating-type cells which triggers a MAP kinase cascade leading to G1 arrest as a prelude to cell fusion. Genetic alteration of certain genes in the pheromone response pathway can alter the normal response to pheromone, and heterologous expression and coupling of human G-protein coupled receptors and humanized G-protein subunits in yeast cells devoid of endogenous pheromone receptors can be linked to downstream signaling pathways and reporter genes (e.g., U.S. Patents 5,063,154; 5,482,835; 5,691,188). Such genetic alterations include, but are not limited to, (i) deletion of the STE2 or STE3 gene encoding the endogenous G-protein coupled pheromone receptors; (ii) deletion of the FAR1 gene encoding a protein that normally associates with cyclin-dependent kinases leading to cell cycle arrest; and (iii) construction of reporter genes fused to the FUS1 gene promoter (where FUS1 encodes a membrane-anchored glycoprotein required for cell fusion).
Downstream reporter genes can permit either a positive growth selection (e.g., histidine prototrophy using the FUS1-HIS3 reporter), or a colorimetric, fluorimetric or spectrophotometric readout, depending on the specific reporter construct used (e.g., β-galactosidase induction using a FUS1-LacZ reporter).

The yeast cells can be further engineered to express and secrete small peptides from random peptide libraries, some of which can permit autocrine activation of heterologously expressed mammalian G-protein coupled receptors (Broach, et al., Nature 384: 14-16, 1996; Manfredi, et al., Mol. Cell. Biol. 16: 4700-4709, 1996). This provides a rapid direct growth selection (e.g., using the FUS1-HIS3 reporter) for surrogate peptide agonists that activate characterized or orphan receptors. Alternatively, yeast cells that functionally express human (or mammalian) G-protein coupled receptors linked to a reporter gene readout (e.g., FUS1-LacZ) can be used as a platform for high-throughput screening of known ligands, fractions of biological extracts and libraries of chemical compounds for either natural or surrogate ligands. Functional agonists of sufficient potency (whether natural or surrogate) can be used as screening tools in yeast cell-based assays for identifying G-protein coupled receptor antagonists. For example, agonists will promote growth of a cell with FUS-HIS3 reporter or give positive readout for a cell with FUS1-LacZ. However, a candidate compound which inhibits growth or negates the positive readout induced by an agonist is an antagonist. For this purpose, the yeast system offers advantages over mammalian expression systems due to its ease of utility and null receptor background (lack of endogenous G-protein coupled receptors) which often interferes with the ability to identify agonists or antagonists.

The present invention also provides a method for identifying new ligands not known to be capable of binding to mHNEAA81 polypeptides. The screening assays described above for identifying agonists may be used to identify new ligands.

The present invention also contemplates agonists and antagonists obtainable from the above-described screening methods.

Examples of potential polypeptide antagonists include antibodies or, in some cases, oligopeptides or proteins that are closely related to ligands, substrates, receptors, enzymes, etc., as the case may be, of a mHNEAA81 polypeptide, e.g., a fragment of a ligand, substrate, receptor, enzyme, etc.; or small molecules which bind to a mHNEAA81
polypeptide but do not elicit a response, so that an activity of a mHNEAA81 polypeptide is prevented.

Thus, in another aspect, the present invention relates to a screening kit for identifying agonists, antagonists, inhibitors, ligands, receptors, substrates, enzymes, etc. for mHNEAA81 polypeptides; or compounds which decrease or enhance the production of such polypeptides, which compounds comprise a member selected from the group consisting of:

(a) a mHNEAA81 polypeptide, preferably that of SEQ ID NO: 2; and further preferably comprises labeled or unlabeled AP4A, AP5A, AP6A, or d-UDP;

(b) a recombinant cell expressing a mHNEAA81 polypeptide, preferably that of SEQ ID NO: 2; and further preferably comprises labeled or unlabeled AP4A, AP5A, AP6A, or d-UDP; or

(c) a cell membrane expressing mHNEAA81 polypeptide; preferably that of SEQ ID NO: 2; and further preferably comprises labeled or unlabeled AP4A, AP5A, AP6A, or d-UDP.

It will be appreciated that in any such kit, (a), (b) or (c) may comprise a substantial component.

As noted above, a potential antagonist is a small molecule which binds to the mHNEAA81 polypeptide receptor, making it inaccessible to ligands such that normal biological activity is prevented. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules.

Potential antagonists also include soluble forms of mHNEAA81 polypeptide receptor; e.g., fragments of the receptor, which bind to the ligand and prevent the ligand from interacting with membrane bound mHNEAA81 polypeptide receptors.

It will also be readily appreciated by the skilled artisan that a mHNEAA81 polypeptide receptor may also be used in a method for the structure-based design of an agonist, antagonist or inhibitor of the polypeptide, by:

(a) determining in the first instance the three-dimensional structure of the polypeptide;
(b) deducing the three-dimensional structure for the likely reactive or binding site(s) of an agonist, antagonist or inhibitor;

(c) synthesizing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and

(d) testing whether the candidate compounds are indeed agonists, antagonists or inhibitors.

It will be further appreciated that this will normally be an iterative process.

In an alternative preferred embodiment, the present invention relates to the use of Mus musculus mHNEAA81 polypeptides, polynucleotides, and recombinant materials thereof in selection screens to identify compounds which are neither agonists nor antagonist/inhibitors of Mus musculus mHNEAA81. The data from such a selection screen is expected to provide in vitro and in vivo comparisons and to predict oral absorption, pharmacokinetics in humans. The ability to make such a comparison of data will enhance formulation design through the identification of compounds with optimal development characteristics, i.e., high oral bioavailability, UID (once a day) dosing, reduced drug interactions, reduced variability, and reduced food effects, among others.

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"AP4A" refers to di-adenosine tetraphosphate.

"AP5A" refers to di-adenosine pentaphosphate.

"AP6A" refers to di-adenosine hexaphosphate.

"d-UDP" refers to deoxy-uridine di-phosphate.

"Allele" refers to one or more alternative forms of a gene occurring at a given locus in the genome.

"Fragment" of a polypeptide sequence refers to a polypeptide sequence that is shorter than the reference sequence but that retains essentially the same biological function or activity as the reference polypeptide. "Fragment" of a polynucleotide sequence refers to a polynucleotide sequence that is shorter than the reference sequence of SEQ ID NO: 1.
"Fusion protein" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0 464 discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved pharmacokinetic properties [see, e.g., EP-A 0232 262]. On the other hand, for some uses, it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected, and purified.

"Homolog" is a generic term used in the art to indicate a polynucleotide or polypeptide sequence possessing a high degree of sequence relatedness to a reference sequence. Such relatedness may be quantified by determining the degree of identity and/or similarity between the two sequences as hereinbefore defined. Falling within this generic term are the terms, "ortholog", and "paralog". "Ortholog" refers to polynucleotides/genes or polypeptide that are homologs via speciation, that is closely related and assumed to have commend descent based on structural and functional considerations. "Paralog" refers to polynucleotides/genes or polypeptide that are homologs via gene duplication, for instance, duplicated variants within a genome.

"Identity" reflects a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, determined by comparing the sequences. In general, identity refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of the two polynucleotide or two polypeptide sequences, respectively, over the length of the sequences being compared. For sequences where there is not an exact correspondence, a "% identity" may be determined. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or very similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.
"Similarity" is a further, more sophisticated measure of the relationship between two polypeptide sequences. In general, "similarity" means a comparison between the amino acids of two polypeptide chains, on a residue by residue basis, taking into account not only exact correspondences between a between pairs of residues, one from each of the sequences being compared (as for identity) but also, where there is not an exact correspondence, whether, on an evolutionary basis, one residue is a likely substitute for the other. This likelihood has an associated 'score' from which the "% similarity" of the two sequences can then be determined.

Methods for comparing the identity and similarity of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J., et al, Nucleic Acids Res, 12, 387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % similarity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (J. Mol. Biol., 147:195-197, 1981, Advances in Applied Mathematics, 2, 482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences that are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP aligns two sequences, finding a "maximum similarity", according to the algorithm of Needleman and Wunsch (J. Mol. Biol., 48, 443-453, 1970). GAP is more suited to comparing sequences that are approximately the same length and an alignment is expected over the entire length. Preferably, the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3, for polynucleotide sequences and 12 and 4 for polypeptide sequences, respectively. Preferably, % identities and similarities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S.F., et al., J. Mol. Biol., 215, 403-410, 1990, Altschul S.F., et al., Nucleic Acids Res., 25:389-3402, 1997, available from the National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA and accessible through the home page of the NCBI at

Preferably, the BLOSUM62 amino acid substitution matrix (Henikoff S. and Henikoff J.G., Proc. Nat. Acad Sci. USA, 89: 10915-10919 (1992)) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Preferably, the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a polynucleotide or a polypeptide sequence of the present invention, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value, as hereinbefore described.

Alternatively, for instance, for the purposes of interpreting the scope of a claim including mention of a "% identity" to a reference polynucleotide, a polynucleotide sequence having, for example, at least 95% identity to a reference polynucleotide sequence is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference sequence. Such point mutations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion. These point mutations may occur at the 5' or 3' terminal positions of the reference polynucleotide sequence or anywhere between these terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a polynucleotide sequence having at least 95% identity to a reference polynucleotide sequence, up to 5% of the nucleotides of the in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same applies mutatis mutandis for other % identities such as 96%, 97%, 98%, 99% and 100%.

For the purposes of interpreting the scope of a claim including mention of a "% identity" to a reference polypeptide, a polypeptide sequence having, for example, at least 95% identity to a reference polypeptide sequence is identical to the reference sequence
except that the polypeptide sequence may include up to five point mutations per each 100 amino acids of the reference sequence. Such point mutations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion. These point mutations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between these terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a sequence polypeptide sequence having at least 95% identity to a reference polypeptide sequence, up to 5% of the amino acids of the in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same applies mutatis mutandis for other % identities such as 96%, 97%, 98%, 99%, and 100%.

A preferred meaning for "identity" for polynucleotides and polypeptides, as the case may be, are provided in (1) and (2) below.

(1) Polynucleotide embodiments further include an isolated polynucleotide comprising a polynucleotide sequence having at least a 95, 97 or 100% identity to the reference sequence of SEQ ID NO:1, wherein said polynucleotide sequence may be identical to the reference sequence of SEQ ID NO:1 or may include up to a certain integer number of nucleotide alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of nucleotides in SEQ ID NO:1, or:

\[ \text{nn} \leq \text{xn} - (\text{xn} \cdot y), \]

wherein nn is the number of nucleotide alterations, xn is the total number of nucleotides in SEQ ID NO:1, y is 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and \( \cdot \) is the symbol for the
multiplication operator, and wherein any non-integer product of \( xn \) and \( y \) is rounded down to the nearest integer prior to subtracting it from \( xn \). Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

(2) Polypeptide embodiments further include an isolated polypeptide comprising a polypeptide having at least a 95, 97 or 100% identity to a polypeptide reference sequence of SEQ ID NO:2, wherein said polypeptide sequence may be identical to the reference sequence of SEQ ID NO:2 or may include up to a certain integer number of amino acid alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of amino acid alterations is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

\[ na \leq xa - (xa \cdot y), \]

wherein \( na \) is the number of amino acid alterations, \( xa \) is the total number of amino acids in SEQ ID NO:2, \( y \) is 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and \( \cdot \) is the symbol for the multiplication operator, and wherein any non-integer product of \( xa \) and \( y \) is rounded down to the nearest integer prior to subtracting it from \( xa \).

"Isolated" means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other
recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

"Knock-in" refers to the fusion of a portion of a wild-type gene to the cDNA of a heterologous gene.

"Knock-out" refers to partial or complete suppression of the expression of a protein encoded by an endogenous DNA sequence in a cell. The "knock-out" can be affected by targeted deletion of the whole or part of a gene encoding a protein, in an embryonic stem cell. As a result, the deletion may prevent or reduce the expression of the protein in any cell in the whole animal in which it is normally expressed.

"Splice Variant" as used herein refers to cDNA molecules produced from RNA molecules initially transcribed from the same genomic DNA sequence but which have undergone alternative RNA splicing. Alternative RNA splicing occurs when a primary RNA transcript undergoes splicing, generally for the removal of introns, which results in the production of more than one mRNA molecule each of that may encode different amino acid sequences. The term splice variant also refers to the proteins encoded by the above cDNA molecules.

"Transgenic animal" refers to an animal to which exogenous DNA has been introduced while the animal is still in its embryonic stage. In most cases, the transgenic approach aims at specific modifications of the genome, e.g., by introducing whole transcriptional units into the genome, or by up- or down-regulating pre-existing cellular genes. The targeted character of certain of these procedures sets transgenic technologies apart from experimental methods in which random mutations are conferred to the germline, such as administration of chemical mutagens or treatment with ionizing solution.

"Polynucleotide” generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. “Polynucleotides” include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, “polynucleotide” refers to
triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs comprising one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may comprise amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well-described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may comprise many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic

“Variant” refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, or deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

All publications including, but not limited to, patents and patent applications, cited in this specification or to which this patent application claims priority, are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.
Examples

Example 1: Mammalian Cell Expression

The receptors of the present invention are expressed in either human embryonic kidney 293 (HEK293) cells or adherent dhfr CHO cells. To maximize receptor expression, typically all 5' and 3' untranslated regions (UTRs) are removed from the receptor cDNA prior to insertion into a pCDN or pCDNA3 vector. The cells are transfected with individual receptor cDNAs by lipofectin and selected in the presence of 400 mg/ml G418. After 3 weeks of selection, individual clones are picked and expanded for further analysis. HEK293 or CHO cells transfected with the vector alone serve as negative controls. To isolate cell lines stably expressing the individual receptors, about 24 clones are typically selected and analyzed by Northern blot analysis. Receptor mRNAs are generally detectable in about 50% of the G418-resistant clones analyzed.

Example 2: Ligand bank for binding and functional assays.

A bank of over 600 putative receptor ligands has been assembled for screening. The bank comprises: transmitters, hormones and chemokines known to act via a human seven transmembrane (7TM) receptor; naturally occurring compounds which may be putative agonists for a human 7TM receptor, non-mammalian, biologically active peptides for which a mammalian counterpart has not yet been identified; and compounds not found in nature, but which activate 7TM receptors with unknown natural ligands. This bank is used to initially screen the receptor for known ligands, using both functional (i.e. calcium, cAMP, microphysiometer, oocyte electrophysiology, etc, see below) as well as binding assays.

Example 3: Ligand Binding Assays

Ligand binding assays provide a direct method for ascertaining receptor pharmacology and are adaptable to a high throughput format. The purified ligand for a receptor is radiolabeled to high specific activity (50-2000 Ci/mmol) for binding studies. A determination is then made that the process of radiolabeling does not diminish the activity of the ligand towards its receptor. Assay conditions for buffers, ions, pH and other modulators such as nucleotides are optimized to establish a workable signal to noise ratio for both membrane and whole cell receptor sources. For these assays, specific receptor binding is defined as total associated radioactivity minus the radioactivity measured in the
presence of an excess of unlabeled competing ligand. Where possible, more than one competing ligand is used to define residual nonspecific binding.

Example 4: Functional Assay in Xenopus Oocytes

Capped RNA transcripts from linearized plasmid templates encoding the receptor cDNAs of the invention are synthesized in vitro with RNA polymerases in accordance with standard procedures. In vitro transcripts are suspended in water at a final concentration of 0.2 mg/ml. Ovarian lobes are removed from adult female toads, Stage V defolliculated oocytes are obtained, and RNA transcripts (10 ng/oocyte) are injected in a 50 nl bolus using a microinjection apparatus. Two electrode voltage clamps are used to measure the currents from individual Xenopus oocytes in response to agonist exposure. Recordings are made in Ca2+ free Barth’s medium at room temperature. The Xenopus system can be used to screen known ligands and tissue/cell extracts for activating ligands.
Example 5: Microphysiometric Assays

Activation of a wide variety of secondary messenger systems results in extrusion of small amounts of acid from a cell. The acid formed is largely as a result of the increased metabolic activity required to fuel the intracellular signaling process. The pH changes in the media surrounding the cell are very small but are detectable by the CYTOSENSOR microphysiometer (Molecular Devices Ltd., Menlo Park, CA). The CYTOSENSOR is thus capable of detecting the activation of a receptor which is coupled to an energy utilizing intracellular signaling pathway such as the G-protein coupled receptor of the present invention.

Example 6: Extract/Cell Supernatant Screening

A large number of mammalian receptors exist for which there remains, as yet, no cognate activating ligand (agonist). Thus, active ligands for these receptors may not be included within the ligands banks as identified to date. Accordingly, the 7TM receptor of the invention is also functionally screened (using calcium, cAMP, microphysiometer, oocyte electrophysiology, etc., functional screens) against tissue extracts to identify natural ligands. Extracts that produce positive functional responses can be sequentially subfractionated until an activating ligand is isolated and identified.

Example 7: Calcium and cAMP Functional Assays

7TM receptors which are expressed in HEK 293 cells have been shown to be coupled functionally to activation of PLC and calcium mobilization and/or cAMP stimulation or inhibition. Basal calcium levels in the HEK 293 cells in receptor-transfected or vector control cells were observed to be in the normal, 100 nM to 200 nM, range. HEK 293 cells expressing recombinant receptors are loaded with fura 2 and in a single day > 150 selected ligands or tissue/cell extracts are evaluated for agonist induced calcium mobilization. Similarly, HEK 293 cells expressing recombinant receptors are evaluated for the stimulation or inhibition of cAMP production using standard cAMP quantitation assays. Agonists presenting a calcium transient or cAMP fluctuation are tested in vector control cells to determine if the response is unique to the transfected cells expressing receptor.
Example 8: mHNEAA81 Receptor Ligand Discovery

HEK-293 cells were transiently co-transfected with a mammalian expression plasmid encoding mHNEAA81 polypeptide, along with cDNA encoding the promiscuous G-protein Go16 and assayed on FLIPR (Fluorometric Imaging Plate Reader) for a calcium mobilization response following addition of AP4A.

A dose-dependent (EC50 ~ 3-5 nM), calcium mobilization response was detected following addition of AP4A to cells transfected with mHNEAA81 and Go16. The agonist nucleotide did not stimulate a calcium mobilization response in HEK-293 cells transfected only with mHNEAA81, nor was a response detected to this ligand in HEK-293 cells transfected only with Go16. The cDNAs for both the receptor and the G-protein had to be expressed in the HEK-293 in order to detect a functional response to these agonists.

Additional G-protein(s) must be present HEK-293 cells in order to detect calcium signaling mediated through mHNEAA81. Thus, in the case of using HEK-293, as described above, additional G-protein(s) is (are) required to run screens for agonists and antagonists. It is possible that mHNEAA81 expressed in another cell, for example RBL-2H3, may signal through calcium pathways without requiring additional G-protein, as has been noted for the C5a receptor (Martino, et al., J. Biol. Chem. 1994 269: 14446-14450), which in some cells also requires additional G-proteins.
SEQUENCE INFORMATION

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What is claimed is:

1. An isolated polynucleotide selected from the group consisting of:
   (i) an isolated polynucleotide comprising a nucleotide sequence encoding a
       polypeptide having at least a 95% identity to the amino acid sequence of SEQ ID
       NO:2, over the entire length of SEQ ID NO:2;
   (ii) an isolated polynucleotide comprising a nucleotide sequence having at least a
       95% identity over its entire length to a nucleotide sequence encoding the polypeptide
       of SEQ ID NO:2;
   (iii) an isolated polynucleotide comprising a nucleotide sequence having at least
       a 95% identity to that of SEQ ID NO:1 over the entire length of SEQ ID NO:1;
   (iv) an isolated polynucleotide comprising a nucleotide sequence encoding the
       polypeptide of SEQ ID NO:2;
   (v) an isolated polynucleotide that is the polynucleotide of SEQ ID NO:1; or
   (vi) an isolated polynucleotide with a nucleotide sequence of at least 100
       nucleotides in length obtained by screening an appropriate library under stringent
       hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1
       or a fragment thereof of at least 15 nucleotides; or a nucleotide sequence
       complementary to said isolated polynucleotide.

2. An isolated polypeptide selected from the group consisting of:
   (i) an isolated polypeptide having at least a 95% identity to the amino acid sequence
       of SEQ ID NO:2 over the entire length of SEQ ID NO:2;
   (ii) an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:2;
   (iii) an isolated polypeptide that is the amino acid sequence of SEQ ID NO:2.

3. A method for screening to identify compounds that stimulate or that inhibit a function or
   level of the polypeptide of Claim 2, comprising a method selected from the group consisting
   of:
(a) measuring or, quantitatively or qualitatively, detecting the binding of a candidate compound to the polypeptide (or to the cells or membranes bearing the polypeptide) or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound;

(b) measuring the competition of the binding of a candidate compound to the polypeptide (or to the cells or membranes bearing the polypeptide) or a fusion protein thereof in the presence of a labeled competitor;

(c) testing whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells or cell membranes bearing the polypeptide;

(d) mixing a candidate compound with a solution comprising a polypeptide of Claim 2, to form a mixture, measuring activity of the polypeptide in the mixture, and comparing the activity of the mixture to a to a control mixture which contains no candidate compound; or

(e) detecting the effect of a candidate compound on the production of mRNA encoding said polypeptide and said polypeptide in cells.

4. An agonist or an antagonist of the polypeptide of Claim 2.

5. An agonist or an antagonist of the Mus musculus mHNEAA81 identified by the method of Claim 3.

6. An expression system comprising a polynucleotide capable of producing a polypeptide of Claim 2 when said expression system is present in a compatible host cell.

7. A process for producing a recombinant host cell comprising the step of introducing the expression system of Claim 6 into a cell, such that the host cell, under appropriate culture conditions, produces said polypeptide.

8. A recombinant host cell produced by the process of Claim 7.
9. A membrane of a recombinant host cell of Claim 8 expressing said polypeptide.

10. A process for producing a polypeptide comprising culturing a host cell of Claim 8 under conditions sufficient for the production of said polypeptide and recovering the polypeptide from the culture.
SEQUENCE LISTING

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SMITHKLINE BEECHAM PLC

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# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

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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. | 536/23.1, 23.5, 24.3, 24.31; 530/350; 435/6, 7.1, 7.2, 471, 325, 252.3, 254.11, 320.1, 71.1, 71.2 |

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

NONE

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


## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

* *A* Special categories of cited documents

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

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

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

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

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

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

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

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

  *S* document member of the same patent family

Date of the actual completion of the international search: 02 January 2001

Date of mailing of the international search report: 21 March 2001

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PREMA MERTZ

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)
A. CLASSIFICATION OF SUBJECT MATTER:
US CL:
536/23.1, 23.5, 24.3, 24.31; 530/350; 435/6, 7.1, 7.2, 471, 325, 252.3, 254.11, 320.1, 71.1, 71.2