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(72) Inventeurs/Inventors:
Weinshank, Richard L., US;
Forray, Carlos, US;
Bard, Jonathan A., US

(73) Propriétaire/Owner:
Synaptic Pharmaceutical Corporation, US

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

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(54) Title: DNA ENCODING HUMAN ALPHA 1 ADRENERGIC RECEPTORS AND USES THEREOF

(57) Abrégé/Abstract:
This invention provides an isolated nucleic acid, vectors, transformed mammalian cells and non-human transgenic animals that encode and express normal or mutant alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention also provides a protein, antibody directed to the protein and pharmaceutical compounds related to alpha 1a, alpha 1b and alpha 1c adrenergic receptors. This invention provides nucleic acid probe, antisense oligonucleotide complementary to alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention further provides methods for determining ligand binding, detecting expression, drug screening, and treatments for alleviating abnormalities associated with human alpha 1a, alpha 1b and alpha 1c adrenergic receptors.

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(71) Applicant *(for all designated States except US):* SYNAPTIC PHARMACEUTICAL CORPORATION [US/US]; 215 College Road, Paramus, NJ 07652 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* BARD, Jonathan, A. [US/US]; 164 Packard Avenue, Wycoff, NJ 07481 (US). FORRAY, Carlos [CL/US]; 7 Birch Lane, Waldwick, NJ 07463 (US). WEINSHANK, Richard, L. [US/US]; 302 West 87th Street, New York, NY 10024 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham, 30 Rockefeller Plaza, New York, NY 10112 (US).

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(54) Title: DNA ENCODING HUMAN ALPHA 1 ADRENERGIC RECEPTORS AND USES THEREOF

(57) Abstract

This invention provides an isolated nucleic acid, vectors, transformed mammalian cells and non-human transgenic animals that encode and express normal or mutant alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention also provides a protein, antibody directed to the protein and pharmaceutical compounds related to alpha 1a, alpha 1b and alpha 1c adrenergic receptors. This invention provides nucleic acid probe, antisense oligonucleotide complementary to alpha 1a, alpha 1b and alpha 1c adrenergic receptor genes. This invention further provides methods for determining ligand binding, detecting expression, drug screening, and treatments for alleviating abnormalities associated with human alpha 1a, alpha 1b and alpha 1c adrenergic receptors.

DNA ENCODING HUMAN ALPHA 1 ADRENERGIC RECEPTORS AND USES THEREOF

Background of the Invention

Throughout this application various publications are referred to by partial citations within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the claims.

Although adrenergic receptors (ARs) bind the same endogenous catecholamines (epinephrine and norepinephrine, NE) their physiological as well as pharmacological specificity is markedly diverse. This diversity is due primarily to the existence of at least nine different proteins encoding three distinct adrenergic receptors types (α_1, $\alpha 2$, and β). These proteins belong to the super-family of G-protein coupled receptors, and are characterized by a single polypeptide chain which span the plasma membrane seven times, with an extracellular amino terminus, and a cytoplasmic carboxyl terminus. The molecular cloning of three genes encoding α_1-ARs supports the existence of pharmacologically and anatomically distinct α_1-receptor subtypes. The α_{1b}-receptor was originally cloned from a hamster smooth muscle cell line cDNA library, and encodes a 515 a.a. peptide that shows 42-47% homology with other ARs. The message for the α_{1b}-receptor is abundant in rat liver, heart, cerebral cortex and kidney, and its gene was localized to human chromosome 5 (4). A second cDNA clone from a bovine brain library was found which encoded a 466-residue polypeptide with 72% homology to the α_{1b}-AR gene. It was further distinguished from α_{1b} by the finding that its expression was restricted to human hippocampus, and

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by its localization to human chromosome 8 and it has been designated as the α_{1c}-AR (20). The cloning of an α_{1a}-AR has been reported recently. This gene, isolated from a rat brain cDNA library, encodes a 560-residue polypeptide that shows 73% homology with the hamster α_{1b}-receptor. The message for this subtype is abundant in rat vas deferens, aorta, cerebral cortex and hippocampus, and its gene has been localized to human chromosome 5 (12).

Pharmacological studies have demonstrated the existence of two α_1-adrenergic receptor subtypes. The studies of α_1-AR-mediated responses in vascular tissue suggested the possible existence of receptor subtypes, based on the potency and efficacy of adrenergic agonists, as well as differential sensitivity of α_1 receptor-mediated responses to extracellular calcium and calcium channel blockers (6, 24). Although radioligand binding studies of brain α_1-ARs with either [^{3}H]WB4101 and [^{3}H]prazosin showed good agreement with the potency of α-adrenergic antagonists on vascular responses (23, 10), subsequent binding studies of rat brain α_1-ARs provided strong evidence for the existence of receptor heterogeneity, based on the relative affinities for prazosin and WB4101 (15). These observations were supported by the finding that chloroethylclonidine (CEC) inactivated 50% of the α_1 sites from rat cerebral cortex and 80% of the binding sites from liver or spleen (α_{1b}), but did not inactivate α_1-receptors from the hippocampus or vas deferens (α_{1a}) (14). Taken together, these results suggested a classification of the α_{1a}-subtype as high affinity for WB4101 and insensitive to alkylation by CEC, and α_{1b}-subtype as 10 to 20 fold lower affinity for WB4101, but sensitive to inactivation by CEC. Consistent with this evidence the transfection of the hamster α_{1b} gene into COS-7 cells induced the expression of an α1-receptor with high

affinity for WB4101, 95% of which could be inactivated by CEC. Conversely, upon expression of the rat α_{1a} receptor gene in COS-7 cells, it showed a 10-fold higher affinity for WB4101 than the α_{1b}-receptor, and the binding site was resistant to inactivation by CEC. The existence of the α_{1c} receptor was not predicted from pharmacological data and upon expression it showed 16 and 30 fold higher affinity for WB4101 and phentolamine respectively, than the α_{1b}-receptor and was partially inactivated (65%) by CEC.

Molecular cloning and pharmacological studies have demonstrated the existence of at least three α_1-adrenergic receptor subtypes. However, it is not clear whether the pharmacological properties of these three cognates might be due also to species differences. This caveat is particularly relevant in the case of the bovine α_{1c} receptor, due to its restricted species and tissue expression. The cloning and expression of the human α_1 adrenergic receptors will allow the further characterization of the pharmacology of the individual human α_1 receptor subtypes.

Summary of the Invention

This invention provides and isolated nucleic acid molecule encoding a human α_1 adrenergic receptor. This invention further provides an isolated nucleic acid molecule encoding a human α_{1a} receptor. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid pcEXV-α_{1a}. This invention also provides an isolated nucleic acid molecule encoding a human α_{1b} receptor. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid pcEXV-α_{1b}. This invention further provides an isolated nucleic acid molecule encoding a human α_{1c} receptor. In one embodiment of this invention, the nucleic acid molecule comprises a plasmid pcEXV-α_{1c}.

This invention also provides vectors such as plasmids comprising a DNA molecule encoding a human α_{1a} receptor, adapted for expression in a bacterial, a yeast cell, or a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or mammalian cells so located relative to the DNA encoding the human α_{1a} receptor as to permit expression thereof. This invention also provides vectors such as plasmids comprising a DNA molecule encoding a human α_{1b} receptor, adapted for expression in a bacterial, a yeast cell, or a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or mammalian cells so located relative to the DNA encoding the human α_{1b} receptor as to permit expression thereof. This invention also provides vectors such as plasmids comprising a DNA molecule encoding a human α_{1c} receptor, adapted for expression in a bacterial, a yeast cell, or a mammalian cell which additionally comprise regulatory elements necessary for expression of the DNA in the bacteria, yeast or mammalian cells so located relative

to the DNA encoding the human α_{1c} receptor as to permit expression thereof.

This invention provides a mammalian cell comprising a DNA molecule encoding a human α_{1a} receptor. This invention also provides a mammalian cell comprising a DNA molecule encoding a human α_{1b} receptor. This invention also provides a mammalian cell comprising a DNA molecule encoding a human α_{1c} receptor.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1a} receptor. This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1b} receptor. This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1c} receptor.

This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an mRNA molecule encoding a human α_{1a} receptor so as to prevent translation of the mRNA molecule. This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an mRNA molecule encoding a human α_{1b} receptor so as to prevent translation of the mRNA molecule. This invention provides an antisense oligonucleotide having a sequence capable of specifically binding to any sequences of an

mRNA molecule encoding a human α_{1c} receptor so as to prevent translation of the mRNA molecule.

This invention provides method for detecting expression of a specific human α_1 adrenergic receptor, which comprises obtaining RNA from cells or tissue, contacting the RNA so obtained with a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_1 receptor under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe, the presence of mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.

This invention provides a method for detecting the expression of a specific human α1 adrenergic receptor in a cell or tissue by in situ hybridization which comprises, contacting the cell or tissue with a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_1 receptor under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe, the presence of mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.

This invention provides a method for isolating a nucleic acid molecule encoding a receptor by nucleic acid sequence homology using a nucleic acid probe, the sequence of which is derived from the nucleic acid

sequence encoding a human α1 adrenergic receptor.

This invention provides a method for isolating a nucleic acid molecule encoding a human α_1 adrenergic receptor which comprises the use of the polymerase chain reaction and oligonucleotide primers, the sequence of which are derived from the nucleic acid sequence encoding a human α1 adrenergic receptor.

This invention provides a method for isolating a human α_1 adrenergic receptor protein which comprises inducing cells to express the human α_1 adrenergic receptor protein, recovering the human α_1 adrenergic receptor from the resulting cells, and purifying the human α_1 adrenergic receptor so recovered.

This invention provides an antibody to the human α_{1a} adrenergic receptor. This invention also provides an antibody to the human α_{1b} adrenergic receptor. This invention also provides an antibody to the human α_{1c} adrenergic receptor.

A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate the abnormalities resulting from overexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention.

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A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention. A pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier is provided by this invention.

This invention provides a transgenic non-human mammal whose genome comprises a nucleic acid molecule encoding a human α1 adrenergic receptor, the DNA molecule so placed as to be transcribed into antisense mRNA complementary to mRNA encoding a human α_1 adrenergic receptor and which hybridizes to mRNA encoding a human α_1 adrenergic receptor thereby reducing its translation.

This invention provides a method for determining the physiological effects of varying the levels of expression of a specific human α1 adrenergic receptor which comprises producing a transgenic non-human mammal whose levels of expression of a human α_1 adrenergic receptor can be varied by use of an inducible promoter.

This invention provides method for determining the physiological effects of expressing varying levels of a specific human α_1 adrenergic receptor which comprises producing a panel of transgenic non-human mammals each

expressing a different amount of the human α_1 adrenergic receptor.

This invention provides a method for determining whether a ligand not known to be capable of specifically binding to a human α_1 adrenergic receptor can bind to a human α_1 adrenergic receptor, which comprises contacting a mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand under conditions permitting binding of ligands known to bind to a human α_1 adrenergic receptor, detecting the presence of any ligand bound to the human α_1 adrenergic receptor, the presence of bound ligand thereby determining that the ligand binds to the human α_1 adrenergic receptor.

This invention provides a method for screening drugs to identify drugs which interact with, and specifically bind to, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with a plurality of drugs, determining those drugs which bind to the human α_1 adrenergic receptor expressed on the cell surface of the mammalian cell, and thereby identifying drugs which interact with, and bind to, the human α_1 adrenergic receptor.

This invention provides a method for identifying a ligand which binds to and activates or blocks the activation of, a human α_1 adrenergic receptor expressed on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises

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a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand, determining whether the ligand binds to and activates or blocks the activation of the receptor using a bioassay such as a second messenger assays.

This invention also provides a method for identifying a ligand which is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, which comprises contacting a mammalian cell, wherein the membrane lipids have been labelled by prior incubation with a labelled lipid precursor molecule, the mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with the ligand and identifying an inositol phosphate metabolite released from the membrane lipid as a result of ligand binding to and activating an α_1 adrenergic receptor.

This invention also provides a method for identifying a ligand that is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, wherein the binding of ligand to the adrenergic receptor results in a physiological response, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with a calcium sensitive fluorescent indicator, removing the indicator that has not been taken up by the cell, contacting the cells with the ligand and identifying an increase or decrease in intracellular Ca^{+2} as a result of ligand binding to and activating or inhibiting α_1 adrenergic receptor activity.

This invention provides a method for detecting the

presence of a human α_{1a} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human $_{\alpha 1a}$ adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α1a adrenergic receptor and thereby the presence of a human α_{1a} adrenergic receptor on the surface of the cell.

This invention provides a method for detecting the presence of a human α_{1b} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human α_{1b} adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α_{1b} adrenergic receptor and thereby the presence of a human α_{1b} adrenergic receptor on the surface of the cell.

This invention provides a method for detecting the presence of a human α_{1c} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody to human α_{1c} adrenergic receptor under conditions which permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the human α_{1c} adrenergic receptor and thereby the presence of a human α_{1c} adrenergic receptor on the surface of the cell.

This invention provides a method of treating an abnormal condition related to an excess of activity of a human α_1 adrenergic receptor subtype, which comprises administering an amount of a pharmaceutical composition effective to reduce α_1 adrenergic activity as a result of naturally occurring substrate binding to and activating a specific α_1 adrenergic receptor.

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This invention provides a method for treating abnormalities which are alleviated by an increase in the activity of a specific human α_1 adrenergic receptor, which comprises administering a patient an amount of a pharmaceutical composition effective to increase the activity of the specific human α_1 adrenergic receptor thereby alleviating abnormalities resulting from abnormally low receptor activity.

This invention provides a method for diagnosing a disorder or a predisposition to a disorder associated with the expression of a specific human α_1 adrenergic receptor allele which comprises: a.) obtaining DNA from subjects suffering from a disorder; b.) performing a restriction digest of the DNA with a panel of restriction enzymes; c.) electrophoretically separating the resulting DNA fragments on a sizing gel; d.) contacting the gel with a nucleic acid probe labelled with a detectable marker and which hybridizes to the nucleic acid encoding a specific human α_1 adrenergic receptor; e.) detecting the labelled bands which have hybridized to the DNA encoding the specific α_1 adrenergic receptor labelled with the detectable marker to create a unique band pattern specific to the DNA of subjects suffering with the disorder; f.) preparing DNA for diagnosis by steps a- e; g.)comparing the unique band pattern specific to the DNA of patients suffering from the disorder from step e and DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from overexpression of a specific human α_1 adrenergic receptor which comprises administering a

substance to the transgenic non-human mammal comprising the DNA encoding a specific α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of the human α_1 adrenergic receptor subtype.

This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor subtype, which comprises administering a substance to a non-human transgenic mammal which is expressing a human α_1 adrenergic receptor incapable of receptor activity or is underexpressing the human α_1 adrenergic receptor subtype, and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of a human α_1 adrenergic receptor subtype.

This invention provides a method of treating abnormalities in a subject, wherein the abnormality is alleviated by the reduced expression of a human α_1 adrenergic receptor subtype which comprises administering to a subject an effective amount of the pharmaceutical composition effective to reduce expression of a specific α_1 adrenergic receptor subtype.

This invention provides a method of treating abnormalities resulting from underexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of a pharmaceutical composition effective to alleviate abnormalities resulting from underexpression of the specific human α_1 adrenergic receptor.

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Brief Description of the Figures

Figures 1A-I. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1a Adrenergic Receptor.

Nucleotides are presented in the 5′ to 3′orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown, along with the 5′ and 3′ untranslated regions. Numbers in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating methionine (M), respectively.

Figures 2A-H. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1b Adrenergic Receptor. Nucleotides are presented in the 5′ to 3′ orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown, along with the 5′ and 3′ untranslated regions. Numbers in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating methionine (M), respectively.

Figures 3A-G. Nucleotide Sequence and Deduced Amino Acid Sequence of Novel Human Alpha-1c Adrenergic Receptor.

Nucleotides are presented in the 5′ to 3′ orientation and the coding region is numbered starting from the initiating methionine and ending in the termination codon. Deduced amino acid sequence by translation of a long open reading frame is shown,

along with the 5′ and 3′ untranslated regions. Numbers in the left and right margins represent nucleotide (top line) and amino acid (bottom line) numberings, starting with the first position as the adenosine (A) and the initiating methionine (M), respectively.

Figures 4A-D. Alignment of the Human Alpha-1a, H318/3 Alpha-1a, and Rat Alpha-1a Adrenergic Receptors.
The deduced amino acid sequence of the human α_{1a} receptor (first line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published human "α_{1a}" adrenergic receptor clone, H318/3 (2)(second line) and with the rat alpha1a (12)(third line). Also shown is a consensus amino acid sequence (fourth line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Note that the human and rat α_{1a} receptors have greater homology in the amino (positions 1-90) and carboxyl (positions 440-598) termini than do the previously published "α_{1a}" (H318/3) and rat α_{1a} receptors (see text). Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid positions, starting with the initiating methionine (M) and ending with the termination codon (*).

Figures 5A-D. Alignment of the Human Alpha-1b, Hamster Alpha-1b, and Rat Alpha-1b Adrenergic Receptors.
The deduced amino acid sequence of the human α_{1b} receptor (third line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published rat α_{1b} adrenergic receptor clone (25)(first line) and with the hamster alpha-1b (4)(second line). Also shown is a consensus amino acid sequence (fourth

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line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid position, starting with the initiating methionine (M) and ending with the termination codon (*).

Figures 6A-C. Alignment of the Human Alpha-1c and Bovine Alpha-1c Adrenergic Receptors.
The deduced amino acid sequence of the human α_{1c} receptor (first line), from the starting methionine (M) to the stop codon (*), is aligned with the previously published bovine α_{1b} adrenergic receptor clone (13)(first line). Also shown is a consensus amino acid sequence (third line), containing a hyphen at a particular position, when all receptors have the same amino acid or an amino acid at this position, when there is disparity in the three receptors. Dots indicate spaces corresponding to no amino acid at this position. Numbers above amino acid sequences correspond to amino acid position, starting with the initiating methionine (M) and ending with the termination codon (*).

Figure 7. Illustrates the correlation of innibition constants (pK_i) for a series of α_1 antagonists at the cloned human α_{1A}, α_{1B}, and α_{1C} receptors with efficiency of blocking contraction of human prostate tissue (pA_2).

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Detailed Description of the Invention

This invention provides an isolated nucleic acid molecule encoding a human α_1 adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1a} adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1b} adrenergic receptor. This invention also provides an isolated nucleic acid molecule encoding a human α_{1c} adrenergic receptor. As used herein, the term "isolated nucleic acid molecule" means a non-naturally occurring nucleic acid molecule that is, a molecule in a form which does not occur in nature. Examples of such an isolated nucleic acid molecule are an RNA, cDNA, or an isolated genomic DNA molecule encoding a human α_{1a}, human α_{1b} or human α_{1c} adrenergic receptor. As used herein, the term "α_{1a} receptor", "α_{1b} receptor" , or " α_{1c} receptor" means a molecule which is a distinct member of a class of α_1 adrenergic receptor molecules which under physiologic conditions, is substantially specific for the catecholamines epinephrine and norepinephrine, is saturable, and having high affinity for the catecholamines epinephrine and norepinephrine. The term "α_1 adrenergic receptor subtype" refers to a distinct member of the class of human α_1 adrenergic receptors, which may be <u>any one</u> of the human α_{1a}, α_{1b} or α_{1c} adrenergic receptors. The term "specific α_1 adrenergic receptor" refers to a distinct member of the group or class of human α_1 adrenergic receptors, which may be any one of the human α_{1a}, α_{1b} or α_{1c} adrenergic receptors. One embodiment of this invention is an isolated human nucleic acid molecule encoding a human α_{1a} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 1A-1I. The DNA molecule of Figures 1A-1I encodes the sequence of the human α_{1a} adrenergic

receptor. Another, preferred embodiment is an isolated human nucleic acid molecule encoding a human α_{1b} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 2A-2H. The DNA molecule of Figures 2A-2H encodes the sequence of the human α_{1b} adrenergic receptor. Another, preferred embodiment is an isolated human nucleic acid molecule encoding a human α_{1c} adrenergic receptor. Such a molecule may have coding sequences substantially the same as the coding sequence in Figures 3A-3G. The DNA molecule of Figures 3A-3G encodes the sequence of the human α_{1c} adrenergic receptor. One means of isolating a nucleic acid molecule encoding a α_1 adrenergic receptor is to screen a genomic DNA or cDNA library with a natural or artificially designed DNA probe, using methods well known in the art. In the preferred embodiment of this invention, α_1 adrenergic receptors include the human α_{1a}, human α_{1b} and human α_{1c} adrenergic receptors and the nucleic acid molecules encoding them were isolated by screening a human genomic DNA library and by further screening of a human cDNA library to obtain the sequence of the entire human α_{1a}, human α_{1b} or human α_{1c} adrenergic receptor. To obtain a single nucleic acid molecule encoding the entire human α_{1a}, α_{1b} or α_{1c} adrenergic receptor two or more DNA clones encoding portions of the same receptor were digested with DNA restriction endonuleases and ligated together with DNA ligase in the proper orientation using techniques known to one of skill in the art. DNA or cDNA molecules which encode a human α_{1a}, α_{1b} or α_{1c} adrenergic receptor are used to obtain complementary genomic DNA, cDNA or RNA from human, mammalian or other animal sources, or to isolate related cDNA or genomic DNA clones by the screening of cDNA or genomic DNA libraries, by methods described in more detail below. Transcriptional regulatory elements from the 5' untranslated region of

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the isolated clone, and other stability, processing, transcription, translation, and tissue specificity determining regions from the 3′ and 5′ untranslated regions of the isolated gene are thereby obtained.

This invention provides an isolated nucleic acid molecule which has been so mutated as to be incapable of encoding a molecule having normal human α_1 adrenergic receptor activity, and not expressing native human α_1 adrenergic receptor. An example of a mutated nucleic acid molecule provided by this invention is an isolated nucleic acid molecule which has an in-frame stop codon inserted into the coding sequence such that the transcribed RNA is not translated into protein.

This invention provides a cDNA molecule encoding a human α_{1a} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 1A-1I. This invention also provides a cDNA molecule encoding a human α_{1b} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 2A-2H. This invention also provides a cDNA molecule encoding a human α_{1c} adrenergic receptor, wherein the cDNA molecule has a coding sequence substantially the same as the coding sequence shown in Figures 3A-3G. These molecules and their equivalents were obtained by the means further described below.

This invention provides an isolated protein which is a human α_1 adrenergic receptor. In one embodiment of this invention, the protein is a human α_{1a} adrenergic receptor having an amino acid sequence substantially similar to the amino acid sequence shown in Figures 1A-IH. In another embodiment of this invention, the protein is a human α_{1b} adrenergic receptor having an

amino acid sequence substantially similar to the amino acid sequence shown in Figures 2A-2H. In another embodiment of this invention, the protein is a human α_{1c} adrenergic receptor having an amino acid sequence substantially similar to the amino acid sequence shown in Figures 3A-3G. As used herein, the term "isolated protein" is intended to encompass a protein molecule free of other cellular components. One means for obtaining an isolated human α_1 adrenergic receptor is to express DNA encoding the α_1 adrenergic receptor in a suitable host, such as a bacterial, yeast, or mammalian cell, using methods well known to those skilled in the art, and recovering the human α_1 adrenergic receptor after it has been expressed in such a host, again using methods well known in the art. The human α_1 adrenergic receptor may also be isolated from cells which express it, in particular from cells which have been transfected with the expression vectors described below in more detail.

This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human α_{1a} receptor. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human human α_{1b} adrenergic receptor. This invention also provides a vector comprising an isolated nucleic acid molecule such as DNA, RNA, or cDNA, encoding a human α_{1c} adrenergic receptor. Examples of vectors are viruses such as bacteriophages (such as phage lambda), cosmids, plasmids (such as pUC18, available from Pharmacia, Piscataway, NJ), and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known to those skilled in the art. Examples of such plasmids are plasmids comprising cDNA having a coding sequence substantially the same as: the coding sequence shown in Figures 1A-

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1I, 2A-2H, and 3A-3G. Alternatively, to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with a ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available.

This invention also provides vectors comprising a DNA molecule encoding a human α_{1a}, vectors comprising a DNA molecule encoding a human α_{1b} adrenergic receptor and vectors comprising a DNA molecule encoding a human α_{1c} adrenergic receptor adapted for expression in a bacterial cell, a yeast cell, or a mammalian cell which additionally comprise the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cells so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. DNA having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I may be inserted into the vectors to express a human α_{1a} adrenergic receptor. DNA having coding sequences substantially the same as the coding sequence shown in Figures 2A-2H may be inserted into the vectors to express a human α_{1b} adrenergic receptor. DNA having coding sequences substantially the same as the coding sequence shown in Figures 3A-3G may be inserted into the vectors to express a human α_{1c} adrenergic receptor. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence

and the start codon AUG (Maniatis, et al., Molecular Cloning, Cold Spring Harbor Laboratory, 1982). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express a human α_1 adrenergic receptor. Certain uses for such cells are described in more detail below.

In one embodiment of this invention a plasmid is adapted for expression in a bacterial, yeast, or, in particular, a mammalian cell wherein the plasmid comprises a DNA molecule encoding a human α_{1a} adrenergic receptor, a DNA molecule encoding a human α_{1b} adrenergic receptor or a DNA molecule encoding a human α_{1c} adrenergic receptor and the regulatory elements necessary for expression of the DNA in the bacterial, yeast, or mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. Suitable plasmids may include, but are not limited to plasmids adapted for expression in a mammalian cell, e.g., pCEXV-3 derived expression vector. Examples of such plasmids adapted for expression in a mammalian cell are plasmids comprising cDNA having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I, 2A-2H, and 3A-3G and the regulatory elements necessary for expression of the DNA in the mammalian cell. These plasmids have been designated pcEXV-α_{1a} deposited under ATCC Accession No. 75319, pcEXV-α_{1b} deposited under ATCC Accession No. 75318, and pcEXV-α_{1c} deposited under ATCC

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Accession No. 75317, respectively . Those skilled in the art will readily appreciate that numerous plasmids adapted for expression in a mammalian cell which comprise DNA encoding human α_1 adrenergic receptors and the regulatory elements necessary to express such DNA in the mammalian cell may be constructed utilizing existing plasmids and adapted as appropriate to contain the regulatory elements necessary to express the DNA in the mammalian cell. The plasmids may be constructed by the methods described above for expression vectors and vectors in general, and by other methods well known in the art.

The deposits discussed supra were made pursuant to, and in satisfaction of, the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852.

This invention provides a mammalian cell comprising a DNA molecule encoding a human α_1 adrenergic receptor, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a human α_1 adrenergic receptor and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding a human α_1 adrenergic receptor as to permit expression thereof. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk$^-$ cells, human embryonic kidney cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, or DNA encoding these human α_1 adrenergic receptors may be otherwise introduced into

mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a human α_1 adrenergic receptor.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1a} adrenergic receptor, for example with a coding sequence included within the sequence shown in Figures 1A-1I. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1b} adrenergic receptor, for example with a coding sequence included within the sequence shown in Figures 2A-2H. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding a human α_{1c} adrenergic receptor, for example with a coding sequence included within the sequence shown in Figures 3A-3G. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. Detection of nucleic acid encoding a human α_1 adrenergic receptor is useful as a diagnostic test for any disease process in which levels of expression of the corresponding human

α_{1a}, α_{1b} or α_{1c} adrenergic receptor are altered. DNA probe molecules are produced by insertion of a DNA molecule which encodes a human α_{1a}, human α_{1b}, or human α_{1c} adrenergic receptor or fragments thereof into suitable vectors, such as plasmids or bacteriophages, followed by insertion into suitable bacterial host cells and replication and harvesting of the DNA probes, all using methods well known in the art. For example, the DNA may be extracted from a cell lysate using phenol and ethanol, digested with restriction enzymes corresponding to the insertion sites of the DNA into the vector (discussed above), electrophoresed, and cut out of the resulting gel. Examples of such DNA molecules are shown in Figures 1A-1I, 2A-2H, and 3A-3G. The probes are useful for "in situ" hybridization or in order to identify tissues which express this gene family, or for other hybridization assays for the presence of these genes or their mRNA in various biological tissues. In addition, synthesized oligonucleotides (produced by a DNA synthesizer) complementary to the sequence of a DNA molecule which encodes a human α_{1a} adrenergic receptor, or complementary to the sequence of a DNA molecule which encodes a human α_{1b} adrenergic receptor or complementary to the sequence of a DNA molecule which encodes a human α_{1c} adrenergic receptor are useful as probes for these genes, for their associated mRNA, or for the isolation of related genes by homology screening of genomic or cDNA libraries, or by the use of amplification techniques such as the Polymerase Chain Reaction.

This invention also provides a method for detecting expression of a human α_{1a} adrenergic receptor on the surface of a cell by detecting the presence of mRNA coding for a human α_{1a} adrenergic receptor. This invention also provides a method for detecting expression of a human α_{1b} adrenergic receptor on the

surface of a cell by detecting the presence of mRNA coding for a human α_{1b} adrenergic receptor. This invention also provides a method for detecting expression of a human α_{1c} adrenergic receptor on the surface of a cell by detecting the presence of mRNA coding for a human α_{1c} adrenergic receptor. These methods comprise obtaining total mRNA from the cell using methods well known in the art and contacting the mRNA so obtained with a nucleic acid probe as described hereinabove, under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of a specific human α_1 adrenergic receptor by the cell. Hybridization of probes to target nucleic acid molecules such as mRNA molecules employs techniques well known in the art. However, in one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the mRNA is isolated from the extract using a column which binds the poly-A tails of the mRNA molecules (Maniatis, T. et al., Molecular Cloning; Cold Spring Harbor Laboratory, pp.197-98 (1982)). The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

This invention provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of an mRNA molecule which encodes a human α_{1a} adrenergic receptor so as to prevent translation of the human α_{1a} adrenergic receptor. This invention also provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of

an mRNA molecule which encodes a human α_{1b} adrenergic receptor so as to prevent translation of the human α_{1b} adrenergic receptor. This invention also provides an antisense oligonucleotide having a sequence capable of specifically binding with any sequences of an mRNA molecule which encodes a human α_{1c} adrenergic receptor so as to prevent translation of the human α_{1c} adrenergic receptor. As used herein, the phrase "specifically binding" means the ability of an antisense oligonucleotide to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. The antisense oligonucleotide may have a sequence capable of specifically binding with any sequences of the cDNA molecules whose sequences are shown in Figures 1A-1I, 2A-2H or 3A-3G. A particular example of an antisense oligonucleotide is an antisense oligonucleotide comprising chemical analogues of nucleotides which are known to one of skill in the art.

This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1a} adrenergic receptor, by passing through a cell membrane and specifically binding with mRNA encoding the human α_{1a} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention also provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1b} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. This invention further provides a pharmaceutical composition comprising an

effective amount of the oligonucleotide described above effective to reduce expression of a human α_{1c} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable hydrophobic carrier capable of passing through a cell membrane. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The oligonucleotide may be coupled to a substance which inactivates mRNA, such as a ribozyme. The pharmaceutically acceptable hydrophobic carrier capable of passing through cell membranes may also comprise a structure which binds to a transporter specific for a selected cell type and is thereby taken up by cells of the selected cell type. The structure may be part of a protein known to bind a cell-type specific transporter, for example an insulin molecule, which would target pancreatic cells. DNA molecules having coding sequences substantially the same as the coding sequence shown in Figures 1A-1I, 2A-2H, or 3A-3G may be used as the oligonucleotides of the pharmaceutical composition.

This invention also provides a method of treating abnormalities which are alleviated by reduction of expression of a human α_1 adrenergic receptor. This method comprises administering to a subject an effective amount of the pharmaceutical composition described above effective to reduce expression of the human α_1 adrenergic receptor by the subject. This invention further provides a method of treating an abnormal condition related to α_1 adrenergic receptor activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to reduce expression of the human $\alpha 1$

adrenergic receptor by the subject. Examples of such an abnormal condition include but are not limited to benign prostatic hypertrophy, coronary heart disease, hypertension, urinary retention, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrythymias erectile dysfunction, and Renaud's syndrome.

Antisense oligonucleotide drugs inhibit translation of mRNA encoding the human α1a, human α1b or human α1c adrenergic receptors. Synthetic antisense oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding the human α1a adrenergic receptor, to mRNA encoding the human α1b adrenergic receptor or to mRNA encoding the human α1c adrenergic receptor and inhibit translation of mRNA and are useful as drugs to inhibit expression of the human α_{1a} adrenergic receptor, the human α_{1b} adrenergic receptor or the human α_{1c} adrenergic receptor in patients. This invention provides a means to therapeutically alter levels of expression of the human α_{1a} adrenergic receptor, the human α_{1b} adrenergic receptor or the human α_{1c} adrenergic receptor by the use of a synthetic antisense oligonucleotide drug (SAOD) which inhibits translation of mRNA encoding these α_1 adrenergic receptors. Synthetic antisense oligonucleotides, or other antisense chemical structures designed to recognize and selectively bind to mRNA, are constructed to be complementary to portions of the nucleotide sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G of DNA, RNA or of chemically modified, artificial nucleic acids. The SAOD is designed to be stable in the blood stream for administration to patients by injection, or in laboratory cell culture conditions, for administration to cells removed from the patient. The SAOD is designed to be capable of passing through cell

membranes in order to enter the cytoplasm of the cell by virtue of physical nd chemical properties of the SAOD which render it capable of passing through cell membranes (e.g., by designing small, hydrophobic SAOD chemical structures) or by virtue of specific transport systems in the cell which recognize and transport the SAOD into the cell. In addition, the SAOD can be designed for administration only to certain selected cell populations by targeting the SAOD to be recognized by specific cellular uptake mechanisms which bind and take up the SAOD only within certain selected cell populations. For example, the SAOD may be designed to bind to a transporter found only in a certain cell type, as discussed above. The SAOD is also designed to recognize and selectively bind to the target mRNA sequence, which may correspond to a sequence contained within the sequences shown in Figures 1A01I, 2A-2H, or 3A-3G by virtue of complementary base pairing to the mRNA. Finally, the SAOD is designed to inactivate the target mRNA sequence by any of three mechanisms: 2) by binding to the target mRNA and thus inducing degradation of the mRNA by intrinsic cellular mechanisms such as mRNA target by interfering with the binding of translation-regulating factors or of other chemical structures, such as ribozyme sequences or reactive chemical groups. which either degrade or chemically modify the target mRNA. Synthetic antisense oligonucleotide drugs have been shown to be capable of the properties described above when directed against mRNA targets (J.S. Cohen, Trends in Pharm. Sci 10, 435 (1989); H.M. Weintraub, Sci. AM. January (1990) p. 40). In addition, coupling of ribozymes to antisense oligonucleotides is a promising strategy for inactivating target mRNA (N. Sarver et al., Science 247, 1222 (1990)). An SAOD serves as an effective therapeutic agent if it is designed to be administered to a patient by injection, or if the patient's target

cells are removed, treated with the SAOD in the laboratory, and replaced in the patient. In this manner, an SAOD serves as a therapy to reduce human α_1 adrenergic receptor expression in particular target cells of a patient, in any clinical condition which may benefit from reduced expression of a specific human α_1 adrenergic receptor.

This invention provides an antibody directed to a human α_{1a} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α_{1a} adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1a} adrenergic receptor included in the amino acid sequence shown in Figures 1A-1I. This invention also provides an antibody directed to a human α_{1b} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α_{1b} adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1b} adrenergic receptor included in the amino acid sequence shown in Figures 2A-2H. This invention also provides an antibody directed to a human α_{1c} adrenergic receptor. This antibody may comprise, for example, a monoclonal antibody directed to an epitope of a human α1c adrenergic receptor present on the surface of a cell, the epitope having an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α1c adrenergic receptor included in the amino acid sequence shown in Figures 3A-3G. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build.

In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer which forms the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Therefore antibodies to the hydrophilic amino acid sequences shown in Figures 1A-1I will bind to a surface epitope of the human α1a adrenergic receptor, antibodies to the hydrophilic amino acid sequences shown in Figures 2A-2H will bind to a surface epitope of a human α1b adrenergic receptor, and antibodies to the hydrophilic amino acid sequences shown in Figures 3A-3G will bind to a surface epitope of a human α1c adrenergic receptor as described. Antibodies directed to human α1 adrenergic receptors may be serum-derived or monoclonal and are prepared using methods well known in the art. For example, monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Cells such as NIH3T3 cells or Ltk$^-$ cells may be used as immunogens to raise such an antibody. Alternatively, synthetic peptides may be prepared using commercially available machines and the amino acid sequence shown in Figures 1A-1I, 2A-2H, and 3A-3G. As a still further alternative DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen. These antibodies are useful to detect the presence of human α1 adrenergic receptors encoded by the isolated DNA, or to inhibit the function of α1 adrenergic receptors in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention provides a pharmaceutical composition which comprises an effective amount of an antibody

directed to an epitope of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1a} adrenergic receptor present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1a} adrenergic receptor included in the amino acid sequence shown in Figures 1A-1I is useful for this purpose. This invention also provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of a human α_{1b} adrenergic receptor, effective to block binding of naturally occurring substrates to the human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1b} adrenergic receptor present on the surface of a cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1b} adrenergic receptor included in the amino acid sequence shown in Figures 2A-2H is useful for this purpose. This invention provides a pharmaceutical composition which comprises an effective amount of an antibody directed to an epitope of a human α_{1c} adrenergic receptor effective to block binding of naturally occurring substrates to the human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier. A monoclonal antibody directed to an epitope of a human α_{1c} adrenergic receptor present on the surface of the cell which has an amino acid sequence substantially the same as an amino acid sequence for a cell surface epitope of the human α_{1c} adrenergic receptor included in the amino acid sequence shown in Figures 3A-3G is useful for this purpose.

This invention also provides a method of treating abnormalities in a subject which are alleviated by reduction of expression of a specific human α_1 adrenergic receptor. The method comprises administering to the subject an effective amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the human α_1 adrenergic receptor and thereby alleviate abnormalities resulting from overexpression of the human α_1 adrenergic receptor. Binding of the antibody to the human α_1 adrenergic receptor from functioning, thereby neutralizing the effects of overexpression. The monoclonal antibodies described above are useful for this purpose. This invention additionally provides a method of treating an abnormal condition related to an excess of a specific human α_1 adrenergic receptor activity which comprises administering to a subject an amount of the pharmaceutical composition described above effective to block binding of naturally occurring substrates to the human α1 adrenergic receptor and thereby alleviate the abnormal condition. Examples of such an abnormal condition include but are not limited to benign prostatic hypertrophy, coronary heart disease, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrythymias, hypertension, urinary retention, erectile dysfunction, and Renaud's syndrome.

This invention provides methods of detecting the presence of a specific human α1 adrenergic receptor on the surface of a cell which comprises contacting the cell with an antibody directed to a specific human α1 adrenergic receptor, under conditions permitting binding of the antibody to the human α1 adrenergic receptor, under conditions permitting binding of the antibody to the human α1 adrenergic receptor, detecting the presence of any antibody bound to the α1 adrenergic

receptor, and thereby the presence of the specific human α1 adrenergic receptor on the surface of the cell. Such methods are useful for determining whether a given cell is defective in expression of a specific human α1 adrenergic receptor. Bound antibodies are detected by methods well known in the art, for example by binding fluorescent markers to the antibodies and examining the cell sample under a fluorescence microscope to detect fluorescence on a cell indicative of antibody binding. The monoclonal antibodies described above are useful for this purpose.

This invention provides a transgenic nonhuman mammal comprising DNA encoding DNA encoding a human α_{1a} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1b} adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1c} adrenergic receptor.

This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1a} adrenergic receptor so mutated as to be incapable of normal human α_{1a} adrenergic receptor activity, and not expressing native human α1a adrenergic receptor activity, and not expressing native human α1a adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1b} adrenergic receptor so mutated as to be incapable of normal human α1b adrenergic receptor activity, and not expressing native human α1b adrenergic receptor. This invention also provides a transgenic nonhuman mammal comprising DNA encoding a human α_{1c} adrenergic receptor so mutated as to be incapable of normal human α1c adrenergic receptor activity, and not expressing native human α1c adrenergic receptor.

This invention provides a transgenic non-human animal whose genome comprises DNA encoding a human α_{1a} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a human α_{1a} adrenergic receptor thereby reducing its translation. This invention also provides a transgenic nonhuman mammal whose genome comprises DNA encoding a human α_{1b} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding the human α_{1b} adrenergic receptor and which hybridizes to mRNA encoding a human α_{1b} adrenergic receptor thereby reducing its translation. This invention provides a transgenic non-human animal whose genome comprises DNA encoding a human α_{1c} adrenergic receptor so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding a human α_{1c} adrenergic receptor and which hybridizes to mRNA encoding the human α_{1c} adrenergic receptor thereby reducing its translation. The DNA may additionally comprise an inducible promoter or additionally comprise tissue specific regulatory elements, so that expression can be induced, or restricted to specific cell types. Examples of DNA are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G. An example of a transgenic animal is a transgenic mouse. Examples of tissue specificity-determining regions are the metallothionein promoter (Low, M.J., Lechan, R.M., Hammer, R.E. et al. Science 231:1002-1004 (1986) and the L7 promoter (Oberdick, J., Smeyne, R.J., Mann, J.R., Jackson, S. and Morgan, J.I. Science 248:223-226 (1990)).

Animal model systems which elucidate the physiological and behavioral roles of human α_1 adrenergic receptors are produced by creating transgenic animals in which the increased or decreased, or the amino acid sequence

of the expressed α_1 adrenergic receptor is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a human α_1 adrenergic receptor or homologous animal versions of these genes, by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal (Hogan B et al., Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)) or, 2) Homologous recombination (Capecchi M.R. Science 244:1288-1292 (1989); Zimmer, A. and Gruss, P. Nature 338:150-153 (1989)) of mutant or normal, human or animal version of the genes encoding α1 adrenergic receptors with the native gene locus in transgenic animals to alter the regulation of expression or the structure α1 of these α1 adrenergic receptors. The technique of homologous α_1 adrenergic receptors. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native α_1 adrenergic receptor but does express, for example an inserted mutant human α_1 adrenergic receptor, which has replaced the native α_1 adrenergic receptor in the animal's genome by recombination, resulting in underexpression of the α_1 adrenergic receptor. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added α_1 adrenergic receptors, resulting in overexpression of the α_1 adrenergic receptor.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B et

al., Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)). DNA or cDNA encoding a human α_1 adrenergic receptor is purified from a vector (such as plasmids pCEXV-α_{1b}, or pCEXV-α_{1c} described above) by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Since the normal action of α_1 adrenergic-specific drugs is to activate or to inhibit the α_1 adrenergic receptor, the transgenic animal model systems described above are useful for testing the biological activity of drugs directed against specific human α_1 adrenergic receptors even before such drugs become available. These animal model systems are useful for predicting or evaluating possible therapeutic applications of drugs which activate or inhibit these human α_1 adrenergic receptors by inducing or inhibiting expression of the native or transgene and thus increasing or decreasing expression of normal or mutant human α_1 adrenergic

receptor in the living animal. Thus, a model system is produced in which the biological activity of drugs directed against these human α_1 adrenergic receptors are evaluated before such drugs become available. The transgenic animals which over or under produce a specific human α_1 adrenergic over or under produce a specific human α_1 adrenergic receptor indicate by their physiological state whether over or under production of the human α_1 adrenergic receptor is therapeutically useful. It is therefore useful to evaluate drug action based on the transgenic model system. One use is based on the fact that it is well known in the art that a drug such as an antidepressant acts by blocking neurotransmitter uptake, and thereby increases the amount of neurotransmitter in the synaptic cleft. The physiological result of this action is to stimulate the production of less human α_1 adrenergic receptor by the affected cells, leading eventually to underexpression. Therefore, an animal which underexpresses human α_1 adrenergic receptor is useful as a test system to investigate whether the actions of such drugs which result in under expression are in fact therapeutic. Another use is that if overexpression is found to lead abnormalities, then a drug which down-regulates or acts as an antagonist to the human α_1 adrenergic receptor is indicated as worth developing, and if a promising therapeutic application is uncovered by these animal model systems, activation or inhibition of the specific human α_1 adrenergic receptor or antagonist drugs directed against these human α_1 adrenergic receptors or by any method which increases or decreases the expression of these α_1 adrenergic receptors in man.

Further provided by this invention is a method of determining the physiological effects of expressing varying levels of a human α_1 adrenergic receptor which

comprises producing a transgenic nonhuman animal whose levels of α_1 adrenergic receptor expression are varied by use of an inducible promoter which regulates human α_1 adrenergic receptor expression. This invention also provides a method for determining the physiological effects of expressing varying levels of human α_1 adrenergic receptors which comprise producing a panel of transgenic nonhuman animals each expressing a different amount of a human α_1 adrenergic receptor. Such animals may be produced by introducing different amounts of DNA encoding a human α_1 adrenergic receptor into the oocytes from which the transgenic animals are developed.

This invention also provides a method for identifying a substance capable of alleviating abnormalities resulting from overexpression of a human α_1 adrenergic receptor comprising administering the substance to a transgenic nonhuman mammal expressing at least one artificially introduced DNA molecule encoding a human α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of a human α_1 adrenergic receptor. As used herein, the term "substance" means a compound or composition which may be natural, synthetic, or a product derived from screening. Examples of DNA molecules are DNA or cDNA molecules having a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H, or 3A-3G.

This invention provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier. This invention

provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier. This invention also provides a pharmaceutical composition comprising an amount of the substance described supra effective to alleviate the abnormalities resulting from overexpression of a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier.

This invention further provides a method for treating the abnormalities resulting from overexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from overexpression of the human α_1 adrenergic receptor.

This invention provides a method for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor comprising administering the substance to the transgenic nonhuman mammal described above which expresses only a nonfunctional human α_1 adrenergic receptor and determining whether the substance alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of the human α_1 adrenergic receptor.

This invention also provides a pharmaceutical composition comprising an amount of a substance effective to alleviate abnormalities resulting from underexpression of a human α_1 adrenergic receptor and a pharmaceutically acceptable carrier.

This invention also provides a method for treating the

abnormalities resulting from underexpression of a human α_1 adrenergic receptor which comprises administering to a subject an amount of the pharmaceutical composition described above effective to alleviate the abnormalities resulting from underexpression of a human α_1 adrenergic receptor.

This invention provides a method for diagnosing a predisposition to a disorder associated with the expression of a specific human α_1 adrenergic receptor allele which comprises: a) obtaining DNA of subjects suffering from the disorder; b) performing a restriction digest of the DNA with a panel of restriction enzymes; c) electrophoretically separating the resulting DNA fragments on a sizing gel; d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing to DNA encoding a human α_1 adrenergic receptor and labelled bands which have hybridized to the DNA encoding a human α^1 adrenergic receptor labelled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; f) preparing DNA obtained for diagnosis by steps a-e; and g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step e and the DNA obtained for diagnosis from step f to determine whether the patterns are the same or different and thereby to diagnose predisposition to the disorder if the patterns are the same. This method may also be used to diagnose a disorder associated with the expression of a specific human α_1 adrenergic receptor allele.

This invention provides a method of preparing an isolated human α_1 adrenergic receptor which comprises inducing cells to express the human α_1 adrenergic receptor, recovering the α_1 adrenergic receptor from the resulting cells, and purifying the α_1 adrenergic

receptor so recovered. An example of an isolated human α_{1a} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in Figures 1A-1I. An example of an isolated human α_{1b} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in Figures 1A-1I. An example of an isolated human α_{1b} adrenergic receptor is an isolated protein having substantially the same amino acid sequence shown in Figure 2A-2H. An example of an isolated human α_{1c} adrenergic receptor is an isolated protein having substantially the same amino acid sequence shown in Figure 3A-3G. For example, cells can be induced to express human α_1 adrenergic receptor by exposure to substances such as hormones. The cells can then be homogenized and the human α_1 adrenergic receptor isolated from the homogenate using an affinity column comprising, for example, epinephrine, norepinephrine, or another substance which is known to bind to the human α_1 adrenergic receptor. The resulting fractions can then be purified by contacting them with an ion exchange column, and determining which fraction contains human α_1 adrenergic receptor activity or binds anti-human α_1 adrenergic receptor activity or binds anti-human $\alpha 1$ adrenergic receptor antibodies.

This invention provides a method of preparing the isolated human α_{1a} adrenergic receptor which comprises inserting nucleic acid encoding the human α_{1a} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host cell, recovering the α_{1a} adrenergic receptor produced by the resulting cell, and purifying the α_{1a} adrenergic receptor so recovered. An example of an isolated human α_{1a} adrenergic receptor is an isolated protein having substantially the same amino acid sequence as the amino acid sequence shown in

Figures 1A-1I. This invention also provides a method of preparing the isolated human α_{1b} adrenergic receptor which comprises inserting nucleic acid encoding the human α_{1b} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host, recovering the α_{1b} adrenergic receptor produced by the resulting cell, and purifying the α_{1c} adrenergic receptor so recovered. These methods for preparing human α_1 adrenergic receptor uses recombinant DNA technology methods well known in the art. For example, isolated nucleic acid encoding a human α_1 adrenergic receptor is inserted in a suitable vector, such as an expression vector. A suitable host cell, such as a bacterial cell, or a eukaryotic cell such as a yeast cell is transfected with the vector. The human α_1 adrenergic receptor is isolated from the culture medium by affinity purification or by chromatography or by other methods well known in the art.

This invention provides a method of determining whether a ligand not known to be capable of binding to a human α_1 adrenergic receptor can bind to a human α_1 adrenergic receptor, which comprises contacting a mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand under conditions permitting binding of ligands known to bind to the human α_1 adrenergic receptor, detecting the presence of any ligand bound to the human α_1 adrenergic receptor. The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2h, or 3A-3G, preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is a Cos7 cell. The preferred method for determining whether a ligand is capable of binding to the human α_1 adrenergic

receptor comprises contacting a transfected nonneuronal mammalian cell (i.e. a cell that does not naturally express any type of human α_1 adrenergic receptor, thus will only express such human α_1 adrenergic receptor if it is transfected into the cell) expressing a human α_1 adrenergic receptor on it surface, or contacting a membrane preparation derived from such a transfected cell, with the ligand under conditions which are known to prevail, and thus be associated with in vivo binding of the substrates to a human α_1 adrenergic receptor, detecting the presence of any of the ligand being tested bound to the human α_1 adrenergic receptor on the surface of the cell, and thereby determining whether the ligand binds to the human α_1 adrenergic receptor. This response system is obtained by transfection of isolated DNA into a suitable host cell. Such a host system might be isolated from pre-existing cell lines, or can be generated by inserting appropriate components into existing cell lines. Such a transfection system provides a complete response system for investigation or assay of the functional activity of human α_1 adrenergic receptors with ligands as described above. Transfection systems are useful as living cell cultures for competitive binding assays between known or candidate drugs and substrates which bind to the human α_1 adrenergic receptor and which are labeled by radioactive, spectroscopic or other reagents. Membrane preparations containing the transporter isolated from transfected cells are also useful for these competitive binding assays. A transfection system constitutes a "drug discovery system" useful for the identification of natural or synthetic compounds with potential for drug development that can be further modified or used directly as therapeutic compounds to activate or inhibit the natural functions of a specific human α_1 adrenergic receptor. The transfection system is also useful for determining the affinity and efficacy of

known drugs at human α_1 adrenergic receptor binding sites.

This invention provides a method for identifying a ligand which interacts with, and activates or blocks the activation of, a human α_1 adrenergic receptor on the surface of the cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with the ligand, determining whether the ligand activates or blocks the activation of the receptor using a bioassay such as a second messenger assays, and thereby identifying a ligand which interacts with, and activates or blocks the activation of, a human α_1 adrenergic receptor.

This invention provides functional assays for identifying ligands and drugs which bind to and activate or inhibit a specific human α1 adrenergic receptor activity.

This invention provides a method for identifying a ligand which is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, which comprises contacting a mammalian cell, wherein the membrane lipids have been labelled by prior incubation with a labelled myo-inositol phosphate molecule, the mammalian cell comprising a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with the ligand and identifying an inositol phosphate metabolite released from the membrane lipid as a result of ligand binding to and activating an α_1 adrenergic receptor.

This invention provides method for identifying a ligand

that is capable of binding to and activating or inhibiting a human α_1 adrenergic receptor, where in the binding of ligand to the adrenergic receptor results in a physiological response, which comprises contacting a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor with a calcium sensitive fluorescent indicator, removing the indicator that has not been taken up by the cell, contacting the cells with the ligand and identifying an increase or decrease in intracellular Ca^{+2} as a result of ligand binding to and activating receptors.

Transformed mammalian cells for identifying the ligands and drugs that affect the functional properties of the human α adrenergic receptor include 292-α1α-10, C-α1b-6 and C-α1c-7.

This invention also provides a method of screening drugs to identify drugs which interact with, and bind to, a human α_1 adrenergic receptor on the surface of a cell, which comprises contacting a mammalian cell which comprises a plasmid adapted for expression in a mammalian cell which further comprises a DNA molecule which expresses a human α_1 adrenergic receptor on the cell surface with a plurality of drugs, determining those drugs which bind to the human α_1 adrenergic receptor expressed on the cell surface of the mammalian cell, and thereby identifying drugs which interact with, and bind to, the human α_1 adrenergic receptor. Various methods of detection may be employed. The drugs may be "labeled" by association with a detectable marker substance (e.g., radiolabel or a non-isotopic label such as biotin). The DNA in the cell may have a coding sequence substantially the same as the coding sequences shown in Figures 1A-1I, 2A-2H or 3A-3G. Preferably, the mammalian cell is nonneuronal in origin. An example of a nonneuronal mammalian cell is

a Cos7 cell. Drug candidates are identified by choosing chemical compounds which bind with high affinity to the human α_1 adrenergic receptor expressed on the cell surface in transfected cells, using radioligand binding methods well known in the art, examples of which are shown in the binding assays described herein. Drug candidates are also screened for selectivity by identifying compounds which bind with high affinity to one particular human α_1 adrenergic receptor subtype but do not bind with high affinity to any other human α_1 adrenergic receptor subtype or to any other known receptor site. Because selective, high affinity compounds interact primarily with the target human α_1 adrenergic site after administration to the patient, the chances of producing a drug with unwanted side effects are minimized by this approach. This invention provides a pharmaceutical composition comprising a drug identified by the method described above and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. Once the candidate drug has been shown to be adequately bio-available following a particular route of administration, for example orally or by injection (adequate therapeutic concentrations must be maintained at the site of action for an adequate period to gain the desired therapeutic benefit), and has been shown to be non-toxic and therapeutically effective in appropriate disease models, the drug may be administered to patients by that route of administration determined to make the drug bio-available, in an appropriate solid or solution formulation, to gain the desired therapeutic benefit.

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This invention also provides a method for treating an abnormal condition related to an excess of activity of a human α_1 adrenergic receptor subtype, which comprises administering a patient an amount of a pharmaceutical composition described above, effective to reduce α_1 adrenergic activity as a result of naturally occurring substrate binding to and activating a specific α_1 adrenergic receptor. Examples of such abnormalities related to an excess of activity of a human α_1 adrenergic receptor subtype include but are limited to benign prostatic hypertrophy, coronary heart disease, hypertension, urinary retention, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrythymias erectile dysfunction, and Renaud's syndrome.

This invention also provides a method of treating abnormalities which are alleviated by an increase in the activity of a specific human α_1 adrenergic receptor, which comprises administering a patient an amount of a pharmaceutical composition described above, effective to increase the activity of the specific human α_1 adrenergic receptor thereby alleviating abnormalities resulting from abnormally low receptor activity. Examples of such abnormalities related to a decrease in the activity of a specific human α_1 adrenergic receptor include but are not limited to congestive heart failure, urinary incontinence, nasal congestion and hypotension.

Applicants have identified individual human α_1 adrenergic receptor subtypes and have described methods for the identification of pharmacological compounds for therapeutic treatments. Pharmacological compounds which are directed against a specific human adrenergic receptor subtype provide effective new therapies with minimal side effects.

Elucidation of the molecular structures of the neuronal human α_1 adrenergic receptors transporters is an important step in the understanding of α-adrenergic neurotransmission. This disclosure reports the isolation, the nucleic acid sequence, and functional expression of DNA clones isolated from human brain which encode human α_1 adrenergic receptor. The identification of these human α_1 adrenergic receptor will play a pivotal role in elucidating the molecular mechanisms underlying α-adrenergic transmission, and should also aid in the development of novel therapeutic agents.

DNA clones encoding human α_1 adrenergic receptor have been isolated from human brain, and their functional properties have been examined in mammalian cells.

This invention identifies for the first time three new human α_1 adrenergic receptor, their amino acid sequences, and their human genes. The information and experimental tools provided by this discovery are useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new human receptors, their associated mRNA molecules or their associated genomic DNAs. The information and experimental tools provided by this discovery will be useful to generate new therapeutic agents, and new therapeutic or diagnostic assays for these new human receptors, their associates mRNA molecules, or their associated genomic DNAs.

Specifically, this invention relates to the first isolation of human DNA clones encoding three α_1-adrenergic receptor. In addition, the human α_1 adrenergic receptor have been expressed in mammalian cells by transfecting the cells with the plasmids pCEXV-α_{1a}, pcEXV-α_{1c}. The pharmacological binding

properties of these receptor proteins have been determined, and these binding properties classify these receptor proteins as α_1 adrenergic receptor. Mammalian cell lines expressing the human α_1 adrenergic receptor on the cell surface have been constructed, thus establishing the first well-defined, cultured cell lines with which to study human α1 adrenergic receptor. Examples of transformed mammalian cells, expressing human α_1 adrenergic receptor are L-α-1a, expressing a human α1a adrenergic receptor, L-α1b expressing a human α1b adrenergic receptor, and L-α1c expressing a human α1c adrenergic receptor. These cells are suitable for studying the pharmacological properties of the human α1 adrenergic receptor and for the screening of ligands and drugs that specifically bind to human α1 adrenergic receptor subtypes.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

MATERIALS AND METHODS

Cloning and Sequencing

α1a: A human lymphocyte genomic library in ζ dash® II (≈1.5 x 10^6 total recombinants; Stratagene, LaJolla, CA.) was screened using a cloned rat PCR fragment (RBNC2) as a probe. RBNC2 was obtained by amplifying randomly primed rat brain cDNA with degenerate primers designed to conserved regions of transmembrane (Tm) regions 2 and 6 of serotonin receptors. The sequence of one PCR product, RBNC2, exhibited strong homology to the α1 AR family.

The probe was labeled with [^{32} P] by the method of random priming (5) (Prime-It® Random Primer kit, Strategene, LaJolla, CA.). Hybridization was performed at 40°C. in a solution containing 50% formamide, 10% dextran sulfate, 5x SSC (1X SSC is 0.15M sodium choloride, 0.015M sodium citrate), 1x Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), and 200 μg/μl sonicated salmon sperm DNA. The filters were washed at 50°C. in 0.1x SSC containing 0.5% sodium dodecyl sulfate and exposed at -70°C to Kodak® XAR film in the presence of an intensifying screen. Lambda phage clones hybridizing with the probe were plaque purified and DNA was prepared for Southern blot analysis (22, 17). For subcloning and further Southern blot analysis, DNA was cloned into pUC18 (Pharmacia, Piscataway, NJ) or pBluescript® (Stratagene, LaJolla, Ca.). Nucleotide sequence analysis was accomplished by the Sanger dideoxy nucleotide chain termination method (18) on denatured double-stranded plasmid templates, using Sequenase (US Biochemcial Corp., Cleveland, OH), Bst DNA sequencing kit (Bio-Rad Laboratories, Richmond, CA.), or TaqTrack® sequencing kit (Promega Corporation, Madison, WI.).

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In order to isolate a full-length clone, human cDNA libraries were screened by polymerase chain reaction (PCR) with 1μM each of specific oligonucleotide primers designed off the isolated genomic clone: from the sense strand (nucleotide 598-626), 5′ CACTCAAGTACCCAGCCATCATGAC 3′ and from the antisense stand (nucleotide 979-1003), 5′ CGGAGAGCGAGCTGCGGAAGGTGTG 3′ (see Figures 1A01I). The primers were from non-conserved portions of the receptor gene, specifically in the Tm3-Tm3 loop and in the Tm5-Tm6 loop regions for the upstream and downstream primers, respectively. One to 2 μl of phage DNA from cDNA libraries (ζ ZapII; Stratagene, LaJolla, CA.),representing ≈10^6-10^7 pfu, were amplified in 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 200 μM each dATP, dCTP, dTTP, 2.5 units of Thermus aquaticus DNA polymerase (Taq polymerase; Perkin-Elmer-Cetus, Norwalk, CT.). The amplification profile was run for 30 cycles: a 5 min. initial (i.e. 1 cycle denaturation at 95°C., followed by 2 min. at 94°C., 2 min at 68°C., and 3 min at 72°C., with a 3 sec. extension, followed by a final 10 min. extension at 72°C. PCR products were analyzed by ethidium bromide (EtBr) stained agarose gels and any sample exhibiting a band on the EtBr stained gel was considered positive.

A positive library was then plated and screened with overlapping 45-mer oligonucleotide probes, filled-in using [α-^{32}P]dCTP and [α-^{32}P]dATP and Klenow fragment of DNA polymerase. This probe was internal to the amplification primers discussed above from the sense strand (nucleotide 890 - 934), 5′ GCAAGGCCTCCGAGGTGGTGCTGCGCATCCACTGTCGCGGCGCGG 3′, and from the anti-sense strand (nucleotide 915-961), 5′ TGCCGTGCGCCCCGTCGGCGCCCGTGGCCGCGCCGCGACAGTGGATG 3′ (see Figures 1A-1I). Positive cDNA phage clones were plaque certified and pBluescript® recombinant DNAs were

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excision-rescued from ζ Zap II using helper phage R408, as described by manufacturer's protocol (Stratagene, LaJolla, CA.). Insert size was confirmed by restriction enzyme digest analysis and recombinants were sequences as described above.

α1b: A human placenta genomic library in λ dash® II (≈1.5 x 10^6 total recombinants; Stratagene, LaJolla, CA.) was screened using overlapping 45-mer oligonucleotides radiolabeled as described above and directed to the third, fifth and sixth transmembrane regions of serotonin 5HT1Dβ receptor gene. Hybridization and washing conditions were identical to that described for α1a above except lower stringency hybridization nd washes were conducted; specifically, hybridization in 25% formamide and washes at 40°C.

Positive-hybridizing λ phage clones were plaque-purified, analyzed by Southern blot analysis, subcloned and sequenced, as described above for α1a. In order to isolate full-length clones, human cDNA libraries in λ Zap II (Strategene, LaJolla, CA.) were screened by polymerase chain reaction as described above. The upstream and downstream PCR primers used were from the Tm40Tm5 loop and the Tm5-Tm6 loop, respectively: from the sense strand (nucleotide 567-593), 5′ CAACGATGACAAGGA GTGCGGGGTCAC 3′, and from the antisense strand (nucleotide 822-847), 5′ TTTGACAGCTATGGAACTCCTGGGG 3′ (see Fig. 2). PCR, library screen, plaque purification excision-rescue from λ Zap II, restriction digestions and sequencing were accomplished as described above for α1a. The internal probe was: from the sense strand (nucleotide 745-789), 5′AAGGAGCTGACCCTGAGGATCCATTCCAAGAACTTTC ACGAGGAC 3′, and from the anti-sense strand (nucleotide 770-814), 5′ CCTTGGCCTTGGTACTGCTAAGGGTGTCCTCGTGAAA GTTCTTGG 3′ (see Figures 2A-2H).

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α1c: A human lymphocyte genomic library in λ dash® II (≈1.5x10^6 total recombinants; Stratagene, LaJolla, CA.) was screened using overlapping 45-mer oligonucleotides radiolabeled as described for α1a and directed to the third, fifth and sixth transmembrane regions of serotonin 5HT1A receptor gene. Hybridization and washing conditions were identical to that described for α1b. Positive-hybridizing λ phage clones were plaque-purified, analyzed by Southern blot analysis, subcloned and sequenced, as described above for α1a. Identification and isolation of full=length clones by PCR and screening cDNA libraries were accomplished as described for α1b. The upstream and downstream PCR primers used were from the Tm3-Tm4 loop and the Tm5-Tm6 loop, respectively: from the sense strand (nucleotide 403-425), 5′ CCAACCATCGTCACCCAGAGGAG 3′, and from the antisense strand (nucleotide 775-802), 5′ TCTCCCGGG AGAACTTGAGGAGCCTCAC 3′ (see Figures 3A-3G). The internal probe was: from the sense strand (nucleotide 711-745), 5′ TCCGCATCCATCGGAAAAACGCCCCGGCAGGAGGC AGCGGGATGG 3′,. and from the anti-sense strand (nucleotide 726-771), 5′ GAAGTGCGTCTTGGTCTTGGCGCT GGCCATCCCGCTGCCTCCTGCC 3′ (see Figures 3A-3G). PCR, library screen, plaque purification excision-rescue from λ Zap II, restriction digestions and sequencing were accomplished as described above for α1a.

Expression

α1a: The entire coding region of α1a (1719 bp), including 150 basepairs of 5′ untranslated sequence (5′ UT) and 300 bp of 3′ untranslated sequence (3′ UT), was cloned into the BamHI and ClaI sites of the polylinker-modified eukaryotic expression vector pCEXV-3 (13), called EXJ.HR (unpublished data). The construct involved the ligation of partial overlapping human lymphocyte genomic and hippocamppal cDNA clones: 5′ sequences were contained on a 1.2 kb SmaI-XhoI genomic

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fragment (the vector-derived BamHI site was used for subcloning instead of the internal insert-derived SmaI site) and 3′ sequences were contained on an 1.3 kb XhoI-ClaI cDNA fragment (the ClaI site was from the vector polylinker). Stable cell lines were obtained by cotransfection with the plasmid α1a/EXJ (expression vector containing the α1a receptor gene) and the plasmid pGCcos3neo (plasmid containing the aminoglycoside transferase gene) into LM(tk$^-$), CHO, NIH3T3 cells, and 293 cells using calcium phosphate technique. The cells were grown, in a controlled environment (37°C., 5% CO_2), as monolayers in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY) containing 25mM glucose and supplemented with 10% bovine calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. Stable clones were then selected for resistance to the antibiotic G-418 (1 mg/ml) as described previously (26) and membranes were harvested and assayed for their ability to bind [^{3}H]prazosin as described below (see "Radioligand Binding Assays").

α1b: The entire coding region of α1b (1563 bp), including 200 basepairs of 5′ untranslated sequence (5′ UT) and 600 bp of 3′ untranslated sequence (3′ UT), was cloned into the EcoRI site of pCEXV-3 eukaryotic expression vector (13). The construct involved ligating the full-length containing EcoRI brainstem cDNA fragment from λ Zap II into the expression vector. Stable cell lines were selected as described above.

α1c: The entire coding region of α1c (1401 bp), including 400 basepairs of 5′ untranslated sequence (5′ UT) and 200 bp of 3′ untranslated sequence (3′ UT), was cloned into the KpnI site of the polylinker-modified pCEXV-3-derived (13) eukaryotic expression vector, EXJ.RH (unpublished data). The construct involved

ligating three partial overlapping fragments: a 5′ 0.6kb HincII genomic clone, a central 1.8 EcoRI hippocamppal cDNA clone, and a 3′ 0.6kb PstI genomic clone. The hippocamppal cDNA fragment overlaps with the 5′ and 3′ genomic clones so that the HincII and PstI sites at the 5′ and 3′ ends of the cDNA clones, respectively, were utilized for ligation. This full-length clone was cloned into the KpnI sites of the fragment, derived from vector (ie pBluescript®)and 3′ untranslated sequences, respectively. Stable cell lines were selected as described above.

Radioligand Binding Assays

Transfected cells from culture flasks were scraped into 5ml of 5mM tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min at 4°C. The pellet was suspended in 50mM Tris-HCl, 1mM $MgCl_2$, and 0.1% ascorbic acid at pH 7.5. Binding of the α1 antagonist [^{3}H]prazosin (0.5 nM, specific activity 76.2 Ci/mmol) to membrane preparations of LM(tk-) cells was done in a final volume of 0.25 ml and incubated at 37°C for 20 min. Nonspecific binding was determined in the presence of 10 μM phentolamine. The reaction was stopped by filtration through GF/B filters using a cell harvester. Data were analyzed by a computerized non-linear regression program.

Measurement of [^{3}H]Inositol Phosphates (IP) Formation

Cells were suspended in Dulbecco's phosphate buffered saline (PBS), and incubated with 5μCi/ml [^{3}H]m-inositol for 60 min at 37°C, the reaction was stopped by adding $CHCl_3$:Methanol: HCl (2/1/0.01 v/v). Total [^{3}H]IP were separated by ion exchange chromatography and quantified as described by Forray and El-Fakahany (7).

Calcium Measurements

Intracellular calcium levels ([Ca^{2+}]i) were determined

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with the calcium-sensitive dye fura-2, and microspectrofluorometry, essentially as previously described (1,3). Briefly, cells were plated into polylysine-coated coverslip bottom dishes (MatTek Corporation, Ashland MA). To lead with fura-2, cells were washed 3x with HEPES-buffered saline (HBS, in mM: HEPES, 20; NaCl, 150; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; pH 7.4) and incubated for 30 minutes at room temperature with fura-2 loading solution (5uM fura-2/AM, 0.03% pluronic® F-127, and 2% heat-inactivated fetal calf serum, in HBS). After loading, cells were washed 3x with HBS, 1ml of HBS was added, and the dish was placed on the microscope for determination of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with a Leitz® Fluovert microscope equipped for UV-transmission epifluorescence. Fura-2 fluorescence was alternately excited at 340 and 380nm (0.25 sec), and a pair of readings (500nm long pass) was taken every two seconds, and recorded by a personal computer interfaced to a data acquisition and control unit from Kinetek (Yonkers, NY). To determine $[Ca^{2+}]_i$ from the experimental data the background fluorescence was subtracted, and the corrected ratios were converted to $[Ca^{2+}]_i$ by comparison with buffers containing saturating and low free calcium, assuming a K_D of 400 nM (3).

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RESULTS

α1a: We screened a human genomic lymphocyte library with a rat PCR fragment that exhibited homology with the α1-AR family. A total of six clones were isolated and characterized by Southern blot analysis. One clone, h13, contained a 4.0kb XbaI fragment which hybridized with the radiolabeled rat PCR fragment and was subsequently subcloned into pUC vector. DNA sequence analysis indicated greatest homology to human α1a and rat α1a ARs. This clone contained the initiating methionine through Tm6 with ≈1.0-1.5kb 5′ UT region. Subsequent Southern blot, analysis, subcloning and sequencing analysis indicated the presence of a SmaI site ≈150nts. 5′ to the initiating methionine codon. The homology between h13 and rat α1a adrenergic gene breaks just downstream of Tm6, indicating an intron which is located in an analogous region in the α1b- and α1c-AR genes (4,20). In order to obtain a full-length clone, aliquots of human cDNA libraries totaling ≈$1.5x10^6$ recombinants was screened by polymerase chain reaction using specific oligonucleotide primers from sequence determined off the genomic clone (see Materials and Methods). A positive- containing human hippocamppal cDNA library (Stratagene, LaJolla, CA.) in λ Zap II (≈$1.5x10^6$ recombinants) was screened using traditional plaque hybridization with an internal probe (see Materials and Methods) and resulted in the isolation of two positive cDNA clones, one containing the upstream sequences (from 5′ UT through the 5-6 loop; hH22) and the other containing downstream sequences (from within Tm5 through ≈200 nts. with a common XhoI site being present within this common region.

The complete full-length gene was constructed by splicing together two restriction fragments, one being the 3′ cDNA (hH14) and the other being the 5′ genomic

clone (h13), using a unique restriction site (XhoI) present in the overlapping region. In addition, another construct was accomplished by ligating the two cDNA clones (hH14 and hH22), using the overlapping XhoI site; however, since this construct produced the same pharmacology as the genomic/cDNA construct, we will not discuss this recombinant (unpublished observation). The genomic/cDNA construct contains an open reading frame of 1719 bp and encoding a protein of 572 aa in length, having a relative molecular mass of ≈63,000 daltons. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family. Initial sequence analysis revealed that clone α1a/EXJ was most related to an AR since it contained a number of conserved structural features/residues found among the members of the adrenergic receptor family, including conserve cysteines in the second and third extracellular loops, a conserved glycine residue in Tm1, aspartic acid residues in Tm regions II and III, conserved valine residues in TmIII, the DRY sequence at the end of TmIII, the conserved proline residues of Tm regions II, IV, V, VI and VII, and the consensus D-V-L-X-X-T-X-S-I-X-X-L-C IN Tm3 and the consensus G-Y-X-N-S-X-X-N-P-X-I-Y in the Tm VII, both consensus unique to the adrenergic receptor family (2,26). Other features of this human α1a receptor gene are the presence of two potential sites for N-linked glycosylation in the amino terminus (asparagine residues 65 and 82; Figures 1a-1I) and the presence of several serines and threonines in the car›xyl terminus and intracellular loops, which may serve as sites for potential phosphorylation by protein kinases.

$\underline{\alpha}_{1b}$: We screened a human genomic placenta library with probes derived from Tm3, 5 and 6 regions of serotonin

5HT1D$_\beta$ under low stringency. Out of several hundred positive clones pursued by Southern blot analysis, subcloning and sequencing, one resembled the α_1 adrenergic family of receptors. This genomic fragment contained Tm3 through Tm6 of a receptor which was most closely related to rat and hamster α_{1b} receptors. In order to obtain a full-length clone, several human cDNA libraries were screened by PCR using primers derived from the 5-6 loop region of the genomic clone (see Materials and Methods). A positive-containing human brainstem cDNA library (Stratagene, LaJolla, CA) in λ ZAPII ($\approx 2 \times 10^6$ recombinants) was screened using traditional plaque hybridization with an internal probe, resulting in the isolation of two identical cDNA clones, containing an insert size of 2.4 kb. Upon sequencing, this clone was found to contain the initiating MET aa, Tm1 through Tm7, and 5′ and 3′ UT sequences, suggesting a full-length clone on a single EcoRI fragment. This cDNA clone contains an open reading frame of 1563 bp and encodes a protein of 520 aa in length, having a relative molecular mass of ≈57,000 daltons. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains, indicative of the G protein-coupled receptor family.

Sequence analysis revealed that clone α_{1b}/pCEXV was most related to adrenergic receptor since it contained a number of conserved structural features found among the adrenergic receptor family, as described for α_{1a} receptor (see above). This human α_{1b} receptor contains potential sites for N-linked glycosylation in the amino terminus (asparagine residues 10, 24, 29, 34 in Fig. 2A-2H), consistent with the finding that the α_1 AR is glycosylated (4,19).

$\underline{\alpha_{1c}}$: We screened a human genomic lymphocyte library

with probes derived from the third, fifth and sixth transmembrane regions of serotonin 5HT1A under low stringency. Out of several hundred positive clones analyzed by Southern blot analysis, subcloning and sequencing (see Materials and Methods), one phage clone resembled a novel α_1 AR. This genomic fragment contained Tm1 through Tm6 of a receptor with high homology to the bovine α_{1c} receptor and thus suggesting the presence of an intron downstream of Tm6, as shown for the α_1 receptor family (4,12,20). In order to obtain a full-length clone, several human cDNA libraries were screened by PCR, as described for α_{1b} (also see Materials and Methods). A positive-containing human hippocamppal cDNA library (Stratagene, LaJolla, CA) in λ ZAPII (≈2 x 10^6 recombinants) was screened, as described for α_{1b}. A positive clone (hH 20) was identified which contained a 1.7kb EcoRI cDNA fragment insert. However, this cDNA clone lacked both the amino end of the receptor (the 5′ end of the clone terminated at the 5′ end of Tm2) and part of the carboxyl tail (the 3′ end of the clone corresponded to 40 aa upstream from the "putative" stop codon). Since an alternative genomic subclone which contained the initiating MET codon in addition to Tm1 through Tm6 was available, we needed to obtain the complete 3′ carboxyl tail in order to complete the construct of the full-length clone. This was accomplished by using overlapping 45-mer oligonucleotide primers (corresponding to nts. 1142-1212 in Fig. 3), designed within the carboxyl tail of the receptor (at the 3′ end of the hH20 cDNA clone), to screen a human lymphocyte genomic library in order to isolate a genomic clone containing the carboxyl tail that includes the termination codon. Two identical positive human lymphocyte genomic clones were isolated from this library. A 0.6 kb PstI fragment was subcloned and shown to contain most of the carboxyl tail (≈20 aa downstream

of Tm7) through the termination codon and ≈200 bp of 3′ UT sequence.

The complete full-length gene was constructed by splicing together three restriction fragments: A 0.6 kb HincII fragment from the genomic clone, containing ≈0.4 kb of 5′ UT sequence and the initiating MET codon through Tm2; the 0.8 kb HincII-PstI fragment from the hH cDNA clone, which contains Tm2 through part of the carboxyl tail, overlapping with the 5′ genomic clone by 20 nts. (sharing the unique HincII site at position 196 in Fig.3); and a 0.6 kb PstI fragment from the second hl genomic clone, which contains the carboxyl tail, the stop codon and ≈0.2 kb of 3′ UT sequence, and overlapping with the hH cDNA clone (sharing the unique Pst I site within the carboxyl tail at position 1038 in Figures 3A-3G).

The resulting genomic/cDNA/genomic construct contains an open reading frame of 1401 bp and encoding a protein of 466 aa in length, having a molecular weight of ≈51,000 daltons. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains, as indicated for the previously described human α_{1a} and α_{1b} receptors and indicative of the G protein-coupled receptor family. Sequence analysis revealed that clone α_{1c}/EXJ was most related to adrenergic receptor because it contained the structural features commonly found among the adrenergic receptor family of receptors, as described for the α_{1a} receptor above. Other features of this human α_{1c} receptor gene is the presence of three potential sites for N-linked glycosylation in the amino terminus, at the same position described for the bovine α_{1c} receptor (asparagine residues 7, 13 and 22 in Figure 3A-3G)(20). Several threonines and serines exist in the second and third cytoplasmic loops of this α_{1c} receptor, which may

serve as potential sites for protein kinases and phosphorylation.

Table 1. Competition of adrenergic agonists and antagonists for the binding of [^{3}H]prazosin to membranes prepared from LM(tk$^-$) cells expressing the human α_{1a}, α_{1b}, and α_{1c}-adrenergic receptor cDNA. Membrane preparations from stably transfected cell lines increasing concentrations of various agonists or antagonists as described under "Materials and Methods". Data is shown as the mean ± S.E.M. of the binding parameters estimated by a computerized non-linear regression analysis obtained in three independent experiments each performed in triplicate.

	pKi		
	α_{1a}	α_{1b}	α_{1c}
AGONISTS			
Norepinephrine	6.633 ± 0.12	5.614 ± 0.09	5.747 ± 0.18
Epinephrine	6.245 ± 0.10	5.297 ± 0.15	5.511 ± 0.13
Oxymetazoline	5.903 ± 0.16	5.919 ± 0.07	7.691 ± 0.10
Naphazoline	6.647 ± 0.18	6.155 ± 0.04	6.705 ± 0.22
Xylometazoline	5.913 ± 0.20	6.096 ± 0.30	7.499 ± 0.19
ANTAGONISTS			
Prazosin	9.479 ± 0.19	9.260 ± 0.23	9.234 ± 0.13
WB-4101	8.828 ± 0.12	7.909 ± 0.13	9.080 ± 0.09
(+) Niguldipine	6.643 ± 0.10	6.937 ± 0.12	8.693 ± 0.18
Indoramin	6.629 ± 0.09	7.347 ± 0.17	8.341 ± 0.25
5-Methyl Urapidil	7.795 ± 0.15	6.603 ± 0.09	8.160 ± 0.11
HEAT	7.857 ± 0.13	8.474 ± 0.10	8.617 ± 0.10
Urapidil	6.509 ± 0.18	5.932 ± 0.11	6.987 ± 0.14

Rauwolscine	5.274 ± 0.12	4.852 ± 0.08	4.527 ± 0.11

Pharmacological Analysis: To further assess the functional identity of the cloned cDNA the coding regions were subcloned into the pCEXV-3 expression vector, and LM(tk-) cell lines stably expressing the human cDNA encoding each of the three α_1-ARs were established. Membrane preparations of these cell lines showed high affinity binding of [^{3}H]prazosin, with Kd values of 0.21 ± 0.03 nM (Bmax= 0.72 ± 0.04 pmol/mg prot), 0.88 ± 0.1 nM (Bmax= 4.59 ± 0.21 pmol/mg prot) and 0.39 ± 0.08 nM (Bmax= 1.9 ± 0.04 pmol/mg prot) for the cells expressing the α_{1a}, α_{1b}, and α_{1c}-ARs respectively. In contrast in competition binding experiments rauwolscine showed extremely low affinity at the three cloned receptors (Table 1), consistent with their identity as α_1-AR. The α-adrenergic agonists NE and epinephrine were found to be 6 and 5-fold respectively, more potent at the human α_{1a}-AR, conversely the imidazoline derivatives such as oxymetazoline and xylometazoline showed 52-fold higher potency at the α_{1c}-AR. Similarly, several antagonists showed marked differences in their potency to inhibit [^{3}H]prazosin binding from the cloned human α_1 receptors subtypes. The selective antagonists WB-4101 and 5-methyl-urapidil showed high affinity for the human α_{1c} subtype (0.8 and 7 nM respectively), followed by less than 2-fold lower potency at the human α_{1a} and at least an order of magnitude (15 and 36-fold respectively) lower potency at the human α_{1b}-AR. Similarly, indoramin was 50 and 10-fold more potent at the α_{1c} than at the α_{1a} and α_{1b} respectively. The calcium channel blocker (+)-niguldipine showed the highest selectivity for the three α_1-AR subtypes, displacing [^{3}H]prazosin 112 and 57-fold more potently from the α_{1c} than from α_{1a} and α_{1b} transfected cells respectively.

Table 2. Receptor-mediated formation of [^{3}H]IP in cell lines transfected with the human α_1-adrenergic receptors cDNA.

Cell lines stably expressing the human α_1-adrenergic receptors were obtained and the IP formation was measured in the absence or presence of 10μM norepinephrine (NE) in the presence of 10 mM LiCl as described under "Material and Methods". Data are shown as mean ± S.E.M. of three independent experiments performed in triplicate.

Cell Line	[^{3}H]IP	Fold Stimulation	Receptor [a] Density
	dpm/dish		pmol/mg Prot
293 α_{1a}			3.30
Control	288 ± 29		
NE	3646 ± 144	13	
CHO α_{1b}			0.49
Control	1069 ± 26		
NE	5934 ± 309	6	
NIH3T3 α_{1c}			0.24
Control	722 ± 61		
NE	13929 ± 1226	19	

[a] Determined by [^{3}H]Prazosin binding.

The formation of [^{3}H]IP was measured in 293, CHO, and

NIH3T3 cell stably expressing the cloned human α_{1a}, α_{1b}, α_{1c}-ARs respectively, to assess the functional coupling of these receptors with the activation of phosphatidyl-inositol specific phospholipase C (PI-PLC). As shown in Table 2, the adrenergic agonist NE (10μM) activated the formation of IP by 13-fold in cells expressing the α_{1a} receptor, and by 5 and 15-fold in cells expressing the α_{1a},α_{1b} and α_{1c} receptors respectively. Furthermore, when cells expressing α_{1b} and α_{1c} receptors were incubated in the presence of 10μM NE, a rapid increase of cytosolic calcium was observed. The response was characterized by an early peak, followed by a plateau that slowly declined towards resting calcium levels (Fig 7). The concentration of $[Ca^{2+}]_i$, was increased by 172 ± 33 (n=6), 170 ± 48 (n=6) and 224 ± 79 nM (n=6) in cell lines transfected with the α_{1a} α_{1b} and α_{1c} receptors respectively. The changes in $[Ca^{2+}]_i$ induced by NE were suppressed by preincubation of the cells with 10 nM prazosin, indicating that the calcium response was mediated by α_1-ARs.

We have cloned DNA representing three α_1-ARs subtypes (α_{1a}, α_{1b} and α_{1c}) from human brain cDNA and genomic DNA. Of all known G protein-coupled receptor sequences (EMBL/Genbank Data Base), the greatest homology was found between α_{1a}/EXJ and the rat α_{1a} AR (12), rat α_{1d} AR (16) and a previously reported putative human "α_{1a}" adrenergic receptor (H318/3)(2). Comparison of the human α_{1a} deduced aa sequence with known α_{1a} ARs indicates the greatest concentration of identical aa to be in the transmembrane domains. In these Tm regions, the percentage of identity for the human α_{1a} AR is 98% compared to rat α_{1a} AR (12) (this is approximately the same for rat α_{1d} since rat α_{1d} AR is the same as rat α_{1a} AR, except for two amino acid differences), 100% with the previously reported H318/3, 78% with the human α_{1b} receptor (see below), and 69% with the human α_{1c}

receptor (see below), which is typical among subtypes. When considering the full-length proteins, the percent identity drops and is only 50% for the human α_{1b} and 49% for the human α_{1c} receptor. Both the alignment (see Fig. 4) and percent identity of this human α_{1a} sequence, relative to other members of the AR family strongly suggest that this is a new receptor and is the human species homolog of the rat α_{1a} receptor.

Figure 4 shows a comparison between the deduced aa sequence of α_{1a}/EXJ and the sequences of rat α_{1a} and HAR. An overall homology of 83.5% aa identity with rat α_{1a} and 86.5% aa identity with the previously published H318/3 clone was observed, suggesting that our human α_{1a} receptor is not any more related to the previously published putative human "α_{1a}" than it is to the rat α_{1a} receptor. In fact, in support of this conclusion, is the fact that the overall aa homology of rat α_{1a} receptor with our human α_{1a} receptor is 83.5% but is only 72% compared to the H318/3 receptor. The main differences between our human α_{1a} receptor and the previously reported "α_{1a}" receptor in relation to the rat α_{1a} are indicated in Fig. 4. Most notably are the differences observed at both the amino and carboxyl ends of the receptor. Specifically, both our human α_{1a} and rat α_{1a} use the starting MET aa at position 1 (see Fig. 4) whereas the previously published H318/3 uses the starting MET 48 aa downstream. Also, the amino terminus of the H318/3 clone is completely divergent from either rat α_{1a} or our human α_{1a} receptor until about 12 aa upstream of Tm1 where significant homology begins. Similarly, in the carboxyl tail, the homology of H318/3 diverges ≈90 aa upstream from the stop codon of either rat or our human α_{1a} receptor and instead, uses a stop codon 30 aa upstream from the stop codon on either of these receptors. Finally, the H318/3 clone has an amino terminal extracellular region that does

not contain potential sites for N-linked glycosylation (2), in contrast to the rat α_{1a} or our human α_{1a} receptor, which contains two potential sites (12, see also Fig. 1 and above). Thus, these data strongly suggest that our human α_{1a} receptor is different in sequence from the previously reported putative human "α_{1a}" (H318/3) but is more related to the previously published rat α_{1a} receptor. Interestingly, the rat α_{1a} aa sequence diverges from both human α_{1a} receptors for ≈65 aa in the carboxyl tail (position 434-508 in Fig. 1); however, homology is seen again in our human α_{1a} receptor but not with H318/3, downstream from this region.

The cloning of different α_1 receptor subtypes permits analysis of both the pharmacological and functional properties of adrenergic receptors. The human α_{1b}/pcEXV clone exhibited the greatest homology with the rat and hamster α_{1b} receptors, out of all known G protein-coupled receptor clones (EMBL/Genbank Data Bank). Comparison of the human α_{1b} deduced aa sequence with known α_1 ARs indicates the greatest homology in the transmembrane regions. In these Tm regions, the percent identity for the human α_{1b} AR is 99% compared to either rat (25) or hamster (4) αb receptor, 78% with human α_{1a} receptor and 75% with human α_{1c} receptor, which is typical among subtypes. When analyzing the full-length proteins, the percent identity slightly drops and is 94.5% compared to rat α_{1b}, 95.5% compared to hamster α_{1b} receptor, 50% compared to human α_{1a} and 51% compared to human α_{1c} receptor. Both the alignment (see Fig. 5) and percent identity of this human α_{1b} sequence, relative to other members of the AR family, strongly suggest that this clone represents a new receptor and is the human species homologue of the rat/hamster α_{1b} receptor. Figure 5 shows a comparison between the deduced amino acid sequence of α_{1b}/pcEXV and

the aa sequence of rat α_{1b} and hamster α_{1b} receptors.

A third human adrenergic receptor clone, α_{1c}/EXJ, showed the greatest homology with the bovine α_{1c} AR gene (20), from all known G protein-coupled receptor sequences (EMBL/Genbank Data Bank). Comparison of the human α_{1c} deduced aa sequence with the α_1 ARs indicates the greatest homology to be in the transmembrane regions. In these Tm regions, the percent identity for the human α_{1c} AR is 97% compared to the bovine α_{1c} AR (20), 75% with human α_{1b} receptor and 69% with human α_{1a} receptor, which is typical among subtypes. When one examines the full-length proteins, the percent identity drops and is only 51% compared to either the human α_{1b} or human α_{1a} receptor. Figure 6 shows a comparison between the deduced amino acid sequence of α_{1c}/EXJ and the aa sequence of bovine α_{1c}. An overall homology of 92% aa identity with bovine α_{1c} receptor was observed. Both the alignment (see Fig. 6) and percent identity of this human α_{1c} sequence, relative to other members of the AR family, strongly suggest that this clone represents a new receptor and is the human species homologue of the bovine α_{1c} receptor.

The stable expression of the three cloned human α_1 receptors enabled the characterization of their pharmacological as well as their functional properties and allowed identification of certain unique features of the human receptors, not predicted from previous data. The rank-order of potency of known α-adrenergic agonists and antagonists to compete with [^{3}H]prazosin in binding assays, confirmed that the cloned cDNAs encode three human receptors of the α_1-AR family. Moreover, the potencies of selective antagonists such as WB-4101 and 5-methyl-urapidil at the three human α_1-receptors were found to be in close agreement with the potencies of these antagonists at the cloned rat α_{1a},

hamster α_{1b}, and bovine α_{1c} (4, 12, 20). These results suggest that the sequence homology between the three mammalian α_1 receptors resulted in a conservation of their pharmacological properties across different species. In the past the pharmacological characterization of α_1-adrenergic receptors took advantage of the existence of selective antagonists such as WB-4101 and 5-methyl-urapidil that bind with high affinity to a subset of α_1-receptors classified as α_{1a} (9, 15). Our results using these selective antagonists indicate that these antagonists bind with similar affinity to both human α_{1a} and α1c-receptors, and that they can only discriminate between either of these two subtypes and the α_{1b} receptor. The calcium channel blocker (+)-niguldipine was found to bind with high affinity to a subset of α_1-receptors also labeled by [^{3}H]5-methyl-urapidil in rat brain, thus defining this antagonist as α_{1a} selective (8). The high affinity of the human α_{1c} receptor for (+)-niguldipine and the fact that it binds to the human α_{1a} and α_{1b} subtypes, with at least an order of magnitude lower affinity, strongly supports the notion that the human α_{1c} gene encodes the pharmacological α_{1a}-receptor subtype. The possibility that this also holds true in the rat, is suggested by the fact that the potency of (+)niguldipine for the rat α_{1a} clone is also at least an order of magnitude lower than that found for this antagonist in rat tissues. Moreover in spite of the earlier reports on the absence of the bovine α_{1c} cognate in rat tissues (20), (24,21) pharmacological evidence suggests that this species express an α_1 receptor similar to the cloned α_{1c} receptor. These data altogether indicate that in trying to match the pharmacological subclassification of the α_1-ARs with the evidence from molecular cloning studies, the initial assignment of the cloned rat α_{1a} receptor with the α_{1a} receptor subtype was inadequate. Recently, a rat

cDNA clone 99.8% homologous to the rat α_{1a}-receptor, was described as a novel α_{1d} subtype (16); however, this incorrect classification was due to the poor correlation between the affinities of α_{1a}-selective antagonists in tissue preparations versus the cloned rat α_{1a} receptor.

The three human α_1 receptor subtypes were able to induce the formation of IP, consistent with the known functional coupling of α_1-ARs, through a GTP-dependent protein to the activation of PI-PLC. In addition we demonstrated that upon receptor activation by adrenergic agonists, the human α_1 subtypes induced transient changes three in $[Ca^{2+}]_i$. Consistent with the mobilization of calcium from intracellular stores by inositol-1,3,5 triphosphate, released by the receptor-mediated activation of PI-PLC.

We have cloned and expressed three human cDNA that encode functional α_1-ARs. These three transcripts display significant pharmacologic as well as molecular features to constitute distinct α_1-AR subtypes. In sharp contrast with the restricted expression of the rat and bovine transcripts, our findings indicate that species homologs of the three α_1-ARs are expressed in human tissues. These findings together with recent reports on the dissimilar tissue distribution of the α_{1b} and α_{1c} receptor cognates between animal species such as rat and rabbit (21), commonly used in the development of novel α_1-adrenergic agents, emphasize the need to study the pharmacological properties of the human α_1-receptors. In this regard, the results from this study on the selectivity of clinically effective antihypertensives such as indoramin, as well as vasoconstrictors such as oxymetazoline and xylometazoline for the human α_{1c}-AR, suggest a potential role for this α_1-receptor subtype in the physiological

control of vascular tone in the human. Thus, the availability of cell lines expressing each of the human α_1-receptor subtypes constitute a unique tool in the design of subtype specific agonists and antagonists, that can be targeted to selective therapeutic applications. Of specific interest for therapeutics are subtype selective alpha-1 antagonists for the treatment of Benign Prostatic Hypertrophy, coronary heart disease, insulin resistance, atherosclerosis, sympathetic dystrophy syndrome, glaucoma, cardiac arrythymias, erectile dysfunction, Reynaud's syndrome, hypertension and urinary retention (44,27,31,32,33,34,35,48). Further interest exists for subtype selective alpha-1 agonists for the treatment of congestive heart failure, nasal congestion, urinary incontinence and hypotension(45,46,47,48). In each case, a more selective drug is expected to reduce the side effects which presently limit this avenue of therapy.

The following compounds were synthesized in order to evaluate their ability to act as antagonists of α_1-receptor function in human prostrate. The synthetic methods used to synthesize are provided herein.

The following Experimental Details are set forth to aid in an understanding of the invention, and are not intended, and should not be construed, to limit in any way the invention set forth in the claims which follow thereafter.

Experimental Details.

Prazosin and 5-methylurapidil were obtained from Research Biochemicals, Inc. A30360 (4-fluoro-4-(8-fluoro-1,3,4,5-tetrahydro-2H-pyrido[4,3-b]indol-2-yl)butyrophenone hydrochloride) was obtained from Aldrich Chemical Co. Other compounds were prepared according to the examples which follow.

Example 1

Synthesis of Terazosin Hydrochloride

N-(2-Furoyl)piperazine

This compound and its preparation has been described in Great Britain Patents 1,390,014 and 1,390,015.

Piperazine hexahydrate (194 g, 1 mole) was dissolved in 250 ml H_2O. The solution was acidified to pH 4.5 with 6 N HCl. Furoyl chloride (130.5 g, 1 mole, Aldrich) was added along with 10% NaOH solution at such a rate that the pH was maintained at 4.5. After 1 hour, the solution was made basic (pH = 8.5) with NaOH solution. The reaction mixture was continuously extracted with chloroform for 36 hours. The $CHCl_3$ extract was dried over $MgSO_4$, and filtered. Distillation gave 108.2 g product (60%), b.p. 132° - 138° C/0.6 mm Hg, m.p. 69° - 70 °C.

N-(Tetrahydro-2-furoyl)piperazine

The furoylpiperazine of Example 1 was converted to the hydrobromide salt (m.p. 173° - 175° C). This salt (39.0 g) in 250 ml methyl alcohol and 9.0 g Raney nickel was hydrogenated at 3 atm. After uptake of H_2 ceased, the catalyst was filtered, the solvent concentrated, and the residue crystallized from isopropyl alcohol to give 35.2 g. tetrahydrofuroylpiperazine HBr, m.p. 152° - 156 °C. This was suspended in 20 ml H_2O. Then 10.5 g 50%, NaOH solution was added slowly followed by 2.0 g solid Na_2CO_3. This was extracted with 4 x 100 ml portions of

warm $CHCl_3$. The $CHCl_3$ extractions were distilled to give 22.5 g tetrahydrofurolylpiperazine, b.p. 120° - 125°C/0.2 mm Hg.

2[4-(Tetrahydro-2-furoyl)piperazinyl]-4-amino-6,7-dimethoxyquinazoline hydrochloride

To 7.00 g 2-chloro-4-amino-6,7-dimethoxyquinazoline (Lancaster Synthesis) in 50 ml methoxyethanol was added 10.8 g, tetrahydrofurolylpiperazine, and the mixture refluxed 3 hours. The clear solution was concentrated and an aqueous solution of potassium bicarbonate was added. The resultant solid that formed was filtered and washed with water. It was then added to methanol and the resulting suspension was acidified with a solution of hydrogen chloride in isopropyl alcohol. The resulting solution was concentrated and the residue crystallized from isopropyl alcohol giving 8.12 g. of product, m.p. 278° - 279°C.

Example 2

Preparation of Indoramin

4-Benzamido-1-[2-(3-indolyl)ethylpyridinium Bromide

A solution of 4-benzamidopyridine (1.98 g) and 3-(2-bromoethyl)indole (2.24 g) in EtOH (15 ml) was refluxed for 2 hours, and the crystallized product (3.13 g, mp 264 - 266°C) was collected by filtration from the hot reaction mixture. Recyrstallization gave the hydrate.

3-[2-4-Benzamidopiperid-1-yl)ethyl]indole (Indoramin)

4-Benzamido-1-[2-(3-indolyl)ethyl]pyridinium bromide (3.0g) in 91% EtOH (300 ml) containing Et_3N (0.8 g) was hydrogenated in the presence of freshly prepared W-7 Raney Ni catalyst (ca. 3 g) at 28.12 kg/cm^2 and 50° for 4 hours. After filtering off the catalyst, the filtrate was evaporated and the residue was shaken with $CHCl_3$ and 2 N NaOH. The resulting insoluble material

(1.61 g, mp 203 - 206°C) was collected and dried. Recrystallization from EtOH gave the product (1.34 g), as colorless needles.

Example 3

Preparation of 1-(3-benzoylpropyl)-4-benzamidopiperidine (Compound 9)

A mixture of 4-chlorobutyrophenone (447 mg, 2.45 mmol), 4-benzamidopiperidine (500 mg, 2.45 mmol) and K_2CO_3 (338 mg, 2.45 mmol) was heated up in boiling water bath for 1 hour. The reaction mixture was portioned between water and $CHCl_3$. The organic layer was separated and dried over Na_2SO_4. After filtration and removal of solvent, the residue was purified by chromatography (SiO_2, MeOH:$CHCl_3$, 5:95). Recrystallization from AcOEt/hexane gave a white powder (78 mg, 8.2%). mp 143-144°C; ^1H NMR (CD_3OD, 400MHz) δ 1.65 (dq, J_1=3.16 Hz, J_2=11.9 Hz, 2H), 1.90-2.00 (m, 4H), 2.18 (t, J=11.9 Hz, 2H), 2.48 (m, 2H), 3.00-3.10 (m, 4H), 3.88 (m, 1H), 7.40-8.00 (m, 10H); Mass spectrum $(M+1)^+$ at m/z 351.

Example 4

Preparation of 1-[3-(4-chlorobenzoyl)propyl]-4-benzamidopiperidine (Compound 7)

A mixture of 3-(4-chlorobenzol)propyl bromide (640 mg, 2.45 mmol), 4-benzamidopiperidine (500 mg, 2.45 mmol) and K_2CO_3 (1.01 g, 7.34 mmol) in 50 ml of acetone was heated up to refluxing condition for 48 hours. The solid was removed by filtration. Concentration of filtrate <u>in vacuo</u> gave a yellowish solid, which was purified by chromatography (SiO_2, MeOH:$CHCl_3$, 5:95). 320 mg (33.9%) of white powder was obtained ^1H NMR ($CDCl_3$, 300 mHz) δ 1.46 (dq, J_1=1.0 Hz, J_2=8.4 Hz, 2H), 1.90-2.10 (m, 4H), 2.16 (m, 2H), 2.43 (t, J=6.9 Hz, 2H), 2.80-2.90 (m, 2H), 2.97 (t, J=6.9 Hz, 2H), 3.97 (m, 1H), 5.92 (d, J=7.8 Hz, 1H, N-H), 7.40-8.00 (m, 9H); Product was converted to HCl salt and

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recrystallized with MeOH/Et_2O, mp 243-244°C; Calcd for $C_{22}H_{25}ClN_2O_2 \cdot HCl \cdot H_2O$: C 60.15, H 6.37, N 6.37; Found: C 60.18, H 6.34, N6.29.

Example 5

Preparation of SKF-104856

1-[(4-Chlorophenyl)thio}-2-propanone

Chloroacetone (32.3 g, 0.347 mol) was added to a mixture of 4-chlorothiophenol (50 g, 0.347 mmol) and sodium hydroxide (14 g, 0.347 mol) in water (400 ml) and the mixture was stirred at 25°C for 1 hour. The mixture was extracted with ethyl ether and the organic phase was washed with water, dried with magnesium sulfate and concentrated to give 69 g (99%) of 1-[(4-chlorophenyl)thio]-2-propanone.

5-Chloro-3-methylbenzo(b)thiophene

1-[(4-Cholorophenyl)thio}-2-propanone (50 g, 0.25 mol) was added to polyphosphoric acid (300 g) and the mixture was stirred as the temperature was gradually raised to 120°C as an exotherm started. The mixture was stirred at 130°C for 1 hour, diluted with water, extracted with ethyl ether and the organic phase was dried and concentrated. The residue was stirred in methanol (200 ml), filtered and the filtrate concentrated to give 17.5 g (40%) of 5-chloro-3-methylbenzo(b)thiophene: bp 120°C (0.6 mm Hg).

Ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate

n-Butyllithium in hexane (2.6 M, 2.3 ml) was added to a solution of 5-chloro-3-methylbenzo(b)thiophene (1,0 g, 6 mmol) in ethyl ether (20 ml) stirred at 0°C under argon. The mixture was stirred for 30 minutes and transferred slowly under argon pressure to a stirred solution of ethyl chloroformate (0.63 g, 6 mmol) in ethyl ether (20 ml). The mixture was stirred at 0°C for 30 minutes and at 25°C for 1.5 hours. The mixture

was treated with water and the organic phase was dried, concentrated and triturated with hexane to give 1.0 g (67%) of ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate: mp 92.5 - 94 °C.

Ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate

A mixture of ethyl 5-chloro-3-methylbenzo(b)thiophene-2-carboxylate (9.0 g, 0.035 mol), N-bromosuccinimide (6.53 g, 0.037 mol) and benzoyl peroxide (130 mg) in carbon tetrachloride (150 ml) was refluxed and illuminated with sunlamp for 2 hours. The resulting suspension was cooled, filtered and the filter cake was triturated with methanol to give 9.9 g, (85%) of the methanol-insoluble ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate: mp 148-150°C.

Ethyl 5-Chloro-3-[N-(2,2-dimethoxyethyl)-N-methyl(aminomethyl)]benzol(b)thiophene-2-carboxylate

A mixture of ethyl 3-bromomethyl-5-chlorobenzo(b)thiophene-2-carboxylate (11 g, 0.033 mol), methylaminoacetaldehyde dimethyl acetal (4.76 g, 0.04 mol) and potassium carbonate (11.4 g, 0.8 mol) in dry acetone (200 ml) was stirred for 48 hours, filtered and the filtrate concentrated to give 11.8 g, (96%) of ethyl 5-chloro-3-(N-2,2-dimethoxyethyl)-N-methyl(aminomethyl)benzol(b)thiophene-2-carboxylate.

Ethyl 7-chloro-3,4-dihydro-4-methylthieno[4,3,2-ef]-[3]benzazepine-2-carboxylate

Ethyl 5-chloro-3-[N-(2,2-dimethoxyethyl)-N-methyl(aminomethyl)]benzo[b]thiophene-2-carboxylate (3.0 g, 8.1 mmol) was added in portions to trifluoromethanesulfonic acid (10 ml) stirred at 0°C under argon. The mixture was stirred at 25°C for 45 minutes and diluted with water. The mixture was basified with aqueous sodium hydroxide and extracted

with ethyl ether to give ethyl 7-chloro-3,4-dihydro-4-methylthieno-[4,3,2-ef][3]benzazepine-2-carboxylate.

Ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate

Diborane in tetrahydrofuaran (1 M, 40 ml) was added to a solution of ethyl 7-chloro-3,4-dihydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate (2.8 g) in tetrahydrofuran (30 ml) stirred at 0°C. The mixture was refluxed for 3 hours and stirred at 25°C for 18 hours, cooled, treated with methanol (50 ml), refluxed for 18 hours and concentrated. The residue was triturated with ethyl ether-hexane (3:1) to give 1.6 g (84%) of ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate: mp 138-140 °C. The free base was treated with hydrogen chloride to give ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-carboxylate hydrochloride: mp 240°C.

7-Chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol

A solution of ethyl 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4.3.2-ef][3]benzazepine-2-carboxylate (4.0 g, 12.9 mmol), in ethyl ether (48 ml) was treated with lithium aluminum hydride (0.53 g, 14 mmol). The mixture was stirred for 1.5 hours, cooled and treated carefully with water (2.0 ml), 10% sodium hydroxide (1.0 ml) and water (2.0 ml). The resulting mixture was filtered and the solvent evaporated to give 1.9 g (57%) of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol: mp 184-185°C.

7-Chloro-3,4,5,6-tetrahydro-4-methylthieno-4,3,2-ef][3]benzazepine-2-carboxaldehyde

A solution of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine-2-methanol (1.6 g,

6 mmol) in dichloromethane (150 ml) was stirred under argon with activated manganese dioxide (8.3 g) for 2 hours. The mixture was filtered through Celite™ and the filtrate was dried with magnesium sulfate and concentrated to give a 63% yield of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef[[3]benzazepine-2-carboxaldehyde.

7-Chloro-2-ethenyl-3,4,5,6-tetrahdyro-4-methylthieno[4,3,2-ef][3]benzazepine (SKF-104856)

Sodium hydride (60 % dispersion in mineral oil. 3.8 mmol) was added to a stirred solution of methyltriphenylphosphonium bromide (1.35 g, 3.8 mmol) in dry tetrahydrofuran (30 ml) and stirred for 15 minutes. The mixture was treated with a solution of 7-chloro-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]-benzazepine-2-carboxaldehyde, prepared as in Example 3, (0.5 g, 1.9 mmol) in dimethylformamide (4 ml), stirred at 25°C for 16 hours, quenched with ice and extracted with ethyl acetate. The organic phase was washed, dried and concentrated and the residue was chromatographed on silica gel eluted with a gradient of methylene chloride to methanol-methylene chloride (3.5:96.5). The product was treated with hydrogen chloride to give 0.2 g (35%) of 7-chloro-2-ethenyl-3,4,5,6-tetrahydro-4-methylthieno[4,3,2-ef][3]benzazepine hydrochloride: mp 234-236°C.

The following is an example of the use of the cloned Human α_1 adrenergic receptors to identify the relevant α_1-Receptor subtype for the therapy of Benign Prostatic Hypertrophy.

Example 6

Protocol for the Determination of the Potency of α_1 Antagonists

The activity of compounds at the different human

receptors was determined in vitro using cultured cell lines that selectively express the receptor of interest. These cell lines were prepared by transfecting the cloned cDNA or cloned genomic DNA or constructs containing both genomic DNA and cDNA encoding the human α-adrenergic, serotonin, histamine, and dopamine receptors as follows:

α_{1A} Human Adrenergic Receptor: The entire coding region of α1A (1719 bp), including 150 basepairs of 5′ untranslated sequence (5′ UT) and 300 bp of 3′ untranslated sequence (3′ UT), was cloned into the BamHI and ClaI sites of the polylinker-modified eukaryotic expression vector pCEXV-3, called EXJ.HR. The construct involved the ligation of partial overlapping human lymphocyte genomic and hippocampal cDNA clones: 5′ sequence were contained on a 1.2 kb SmaI-XhoI genomic fragment (the vector-derived BamHI site was used for subcloning instead of the internal insert-derived SmaI site) and 3′ sequences were contained on an 1.3 kb XhoI-ClaI cDNA fragment (the ClaI site was from the vector polylinker). Stable cell lines were obtained by cotransfection with the plasmid α1A/EXJ (expression vector containing the α1A receptor gene) and the plasmid pGCcos3neo (plasmid containing the aminoglycoside transferase gene) into LM(tk^-), CHO, and NIH3T3 cells, using calcium phosphate technique. The cells were grown, in a controlled environment (37°C., 5% CO_2), as monolayers in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY) containing 25mM glucose and supplemented with 10% bovine calf serum, 100 units/ml penicillin g, and 100 μg/ml streptomycin sulfate. Stable clones were then selected for resistance to the antibiotic G-418 (1 mg/ml), and membranes were harvested and assayed for their ability to bind [^{3}H]prazosin as described below (see "Radioligand Binding assays").

α_{1B} Human Adrenergic Receptor: The entire coding region of α1B (1563 bp), including 200 basepairs and 5′ untranslated sequence (5′ UT) and 600 bp of 3′ untranslated sequence (3′ UT), was cloned into the EcoRI site of pCEXV-3 eukaryotic expression vector. The construct involved ligating the full-length containing EcoRI brainstem cDNA fragment from λ ZapII into the expression vector. Stable cell lines were selected as described above.

α_{1C} Human Adrenergic Receptor: The entire coding region of α1C (1401 bp), including 400 basepairs of 5′ untranslated sequence (5′ UT) and 200 bp of 3′ untranslated sequence (3′ UT), was cloned into the KpnI site of the polylinker-modified pCEXV-3-derived eukaryotic expression vector, EXJ.RH. The construct involved ligating three partial overlapping fragments: a 5′ 0.6kb HincII genomic clone, a central 1.8 EcoRI hippocampal cDNA clone, and a 3′ 0.6Kb PstI genomic clone. The hippocampal cDNA fragment overlaps with the 5′ and 3′ genomic clones so that the HincII and PstI sites at the 5′ and 3′ ends of the cDNA clone, respectively, were utilized for ligation. This full-length clone was cloned into the KpnI site of the expression vector, using the 5′ and 3′ KpnI sites of the fragment, derived from vector (i.e., pBluescript) and 3′-untranslated sequences, respectively. Stable cell lines were selected as described above.

Radioligand Binding Assays: Transfected cells from culture flasks were scraped into 5ml of 5mM Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min at 4°C, and the supernatant was centrifuged at 30,000 x g for 20 min at 4°C. The pellet was suspended in 50mM Tris-HCl, 1mM $MgCl_2$, and 0.1% ascorbic acid at pH 7.5. Binding of the α1 antagonist [^{3}H]prazosin (0.5 nM,

specific activity 76.2 Ci/mmol) to membrane preparations of LM(tk-) cells was done in a final volume of 0.25 ml and incubated at 37°C for 20 min. Nonspecific binding was determined in the presence of 10 μM phentolamine. The reaction was stopped by filtration through GF/B filters using a cell harvester. Inhibition experiments, routinely consisting of 7 concentrations of the tested compounds, were analyzed using a non-linear regression curve-fitting computer program to obtain Ki values.

Example 7

Functional Properties of α_1 Antagonists in the Human Prostate

The efficacy of α_1 adrenergic antagonists for the treatment of benign prostatic hyperplasia (BPH) is related to their ability to elicit relaxation of prostate smooth muscle. An index of this efficacy can be obtained by determining the potency of α_1 antagonists to antagonize the contraction of human prostatic tissue induced by an α_1 agonist "in vitro". Furthermore, by comparing the potency of subtype selective α_1 antagonists in binding assays using human α_1 receptors with their potency to inhibit agonist-induced smooth muscle contraction, it is possible to determine which of the α_1 adrenergic receptor subtypes is involved in the contraction of prostate smooth muscle.

Methods: Prostatic adenomas were obtained at the time of surgery from patients with symptomatic BPH. These were cut into longitudinal strips of 15mm long and 2-4 mm wide, and suspended in 5ml organ baths containing Krebs buffer (pH 7.4). The baths were maintained at 37°C and continuously oxygenated with 5% CO_2 and 95% O_2. Isometric tension was measured with a Grass Instrument FT03 force transducer interfaced with a computer.

Tissue strips were contracted with varying concentrations of phenylephrine after incubating for 20 minutes in the absence and presence of at least three different concentrations of antagonist. Dose-response curves for phenylephrine were constructed, and the antagonist potency (pA_2) was estimated by the dose-ratio method. The concentration of some antagonists in the tissue bath was assessed by measuring the displacement of [3H]prazosin by aliquots of the bath medium, using membrane preparations of the cloned human α_{1C} receptor. This control was necessary to account for losses of antagonist due to adsorption to the tissue bath and/or metabolism during the time the antagonists were equilibrated with the prostate tissue.

Results:

Table 3 shows that the pA_2 values measured for a series of α_1 antagonists in human prostate tissue correlate closely (r=0.76) with the corresponding pK_i values measured in the α_{1C} receptor assays. In contrast, the human prostate pA_2 values correlate poorly with the pK_i values measured at the α_{1A} (r=-0.06) and α_{1B} (r=-0.24) adrenergic receptors. (See Figure 7.) Thus, antagonists which are more potent at blocking the α_{1C} adrenergic receptor are more effective at blocking the contraction of the human prostate than antagonists which are more potent at the α_{1A} or α_{1B} adrenergic receptors. In addition, antagonists which are selective for the α_{1C} receptor will have a better therapeutic ratio than nonselective α antagonists.

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Table 3.

COMPARISON OF THE BINDING POTENCY (pK_i) OF ALPHA-1 ANTAGONISTS IN CLONED HUMAN RECEPTORS AND THEIR PROTENCY (pA_2) TO INHIBIT PROSTATE SMOOTH MUSCLE CONTRACTION

	Compound	Human Alpha-1 Adrenergic (pK_i)			Human Prostate (pA)
		a1A	a1B	a1C	
1	Prazosin	9.48	9.26	9.23	9.08
3	A-30360	7.49	7.86	8.52	8.72
4	5-Methyl-Urapidil	7.79	6.77	8.35	8.38
5	Indoramin	6.74	7.39	8.35	7.86
6	SKF-104856	8.48	7.50	7.60	7.66
7	Compound 7	6.82	7.18	8.42	7.63
9	Compound 9	6.12	6.76	7.83	7.41
10	Terazosin	8.46	8.71	8.16	7.30

References

1.- Borden, L.A., Maxfield, F.R., Goldman, J.E., and Shelanski, M.L., Neurobiol. Aging., 13, 33-38, 1991.

2.- Bruno, J. F., J. Whittaker, J. Song, and M. Berelowitz. Biochem. Biophys. Res. Comm. 179, 1485-1490 (1991).

3.- Bush, A.W., Borden, L.A., Greene, L.A., and Maxfield, F.R., J. Neurochem. 57, 562-574, 1991.

4.- Cotecchia, S., Schwinn, D.A., Randall, R.R., Lefkowitz, R.J., Caron, M.G., and Kobilka, B.K., Proc. Natl. Acad. Sci. USA, 85, 7159-7163, 1988.

5.- Feinberg, A. P., and B. Vogelstein. Anal. Biochem. 132, 6-13 (1983).

6.- Flavahan, N.A. and Vanhoutte, P.M., In: The Alpha-1 Adrenergic Receptors, (ed. by R.R. Ruffolo, Jr., Humana Press, Clifton NJ) pp. 351-403, 1987.

7.- Forray, C., and El-Fakahany, E.E., Mol. Pharmacol., 37, 893-902, 1990.

8.- Graziadei, I., Zernig, G., Boer, R., and Glossman, H., Eur. J. Pharmacol. 172, 329-337, 1989.

9.- Gross, G., Hanft, G., and Rugevics, C., Eur. J. Pharmacol., 151,333-335, 1989.

10.- Hieble, J.P., Sarau, H.M., Foley, J.J., DeMarinis, R.M., and Pendleton, P.G., Naunyn-Schmiedeberg's Arch. Pharmacol., 318, 267-273, 1982.

11.- Langer, S.Z., Pharmacol. Rev., 32, 377-360, 1980.

12.- Lomasney, J.W., Cotecchia, S., Lorenz, W., Leung, W.-Y., Schwinn, D.A., Yang-Feng, T.L., Brownstein, M., Lefkowitz, R.J., and Caron, M., J. Biol. Chem., 266, 6365-6369, 1991.

13.- Miller, J. and R. N. Germain. J. Exp. Med. 164, 1478-1489 (1986).

14.- Minneman, K.P., Pharmacol. Rev., 40, 87-119, 1988.

15.- Morrow, A.L., and Creese, I., Mol. Pharmacol., 29, 321-330, 1986.

16.- Perez, D. M., M. T. Piascik, and R. M. Graham. Mol. Pharmacol. 40, 876-883 (1991).

17.- Sambrook, J., Fritsch, E.F., and Maniatis, T., In: Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.), 1989.

18.- Sanger, S. Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977).

19.- Sawutz, D. G., S. M. Lanier, C. D. Warren, and R. M. Graham. Mol. Pharmacol. 32, 565-571 (1987).

20.- Schwinn, D.A., Lomasney, J.W., Lorenz, W., Szklut, P.J., Fremeau, R.T., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J. and Cotecchia, S., J. Biol. Chem., 265, 8183-8189, 1990.

21.- Schwinn, D.A., Page, S.A., Middleton, J.P., Lorenz, W., Liggett, S.B., Yamamoto, E., Lapetina, E.G., Caron, M.G., Lefkowitz, R.J., and Cotecchia,

S., Mol. Pharmacol., 40, 619-626, 1991.

22.- Southern, E. M. J. Mol. Biol. 98,503-505 (1975).Starke, S., Rev. Physiol. Biochem. Pharmacol., 88, 199-236, 1981.

23.- Timmermans, P.B.M.W.M., Karamat Ali, F., Kwa, H.Y.,Schoop, A.M.C., Slothorst-Grisdijk, F.P., and van Zwieten, P.A., Mol. Pharmacol., 20, 295-301, 1981.

24.- Timmermans, P.B.M.W.M., and Thoolen. M.J.M.C., In: The Alpha-1 Adrenergic Receptors, (ed. by R.R. Ruffolo, Jr., Humana Press, Clifton NJ) pp. 113-187, 1987.

25.- Voigt, M. M., J. Kispert, and H. Chin. Nucleic Acid Res. 18, 1053 (1990).

26.- Weinshank, R.L., Zgombick, J.M., Macchi, M., Adham, N., Lichtblau, H., Branchek, T.A., and Hartig, P.R., Mol. OPharmacol., 38, 681-688, 1990.

27.- Cohen, J., (1993) **J. Clin. Pharmacol.**, 33, 272-278.

28.- Manning, A.S. and Hearse, D.J., (1984) **J. Mol. Cell Cardiol.**, 16: 497-518.

29.- Benfey, B.G., (1992) **Can. J. Physiol. Pharmacol.**, 71: 103-111.

30.- Cubeddu, L. X., (1984) **American Heart Journal**, 116: 133-161.

31.- Nishimura, K., Kuwayama, Y., Matsugi, T., Sun, N., and Shirasawa, E., (1993) **Investigative Ophthal. & Visual Sci.**, 34: 1761-1765.

32.- Kincaid-Smith, P., (1989) **Journal of Human Hypertension**, 2743: 75-83.

33.- Ames, R. P. and Kiyasu, J.Y., (1989) **J. Clin. Pharmacol.**, 29: 123-127.

34.- Pool, J.L., (1991) **Am. Heart J.**, 121: 251-260.

35.- Christ, G.J., Schwartz, C.B., Stone, B.A., Parker, M., Janis, M., Gondre, M., Valcic, M., and Melman, A., (1992) **Amer. Physiological Soc.**, H15-H20.

36.- Rosenthal, J., (1989) **Journal of Human Hypertension**, 3: 85-91.

37.- Kowala, M.C. and Nicolosi, R.J., (1989) **Journal of Cardiovascular Pharm.**, 13: 545-549.

38.- Nash, D.T., (1990) **Clin. Cardiol.**, 13: 764-772.

39.- Waite, M.A., (1991) **Journal of Internal Medicine**, 229: 113-117.

40.- Achari, R. and Laddu, A., (1992) **J. Clin. Pharm.**, 32: 520-523.

41.- Kowala, M.C., Nunnari, J.J., Durham, S.K., and Nicolosi, R.J., (1991) **Atherosclerosis**, 91: 35-39.

42.- Krupp, M.N., Hoover, K.W., and Valentine, J.J., (1989) **Journal of Cardiovascular Pharm.**, 13: 511-519.

43.- Jansen, H., Lammers, R., Baggen, M.G.A., and Birkenhäger, J.C., (1989) **Journal of Cardiovascular Pharm.**, 13: S5-S10.

44.- Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure, (1993) **Archives of Internal Medicine**, 163: 164-183.

45.- Sourander, L.B., (1990) **Gerontology**, 36: 19-25.

46.- Empey, D.W. and Medder, K.T., (1988) **Drugs**, 35: 438-443.

47.- Andersson, K.E., (1988) **Drugs**, 35: 477-494.

48.- MacDonald, E., Ruskoaho, H., Scheinen, M. and Virtanen, R., (1988) **Annals of Clin. Res.**, 20: 298-310.

49.- Lepor, H., R. Tan, S. Meretyk, and E. Shapiro. Alpha$_1$ adrenoceptor subtypes in the human prostate. J. Urol. 149:640-642 (1993).

50.- Lepor, H., S. Auerbach, A Puras-Baez, P. Narayan, M. Soloway, F. Lowe, T. Moon, G. Leifer, and P. Madsen. A randomized, placebo-controlled multicenter study of the efficacy and safety of teazosin in the treatment of benign porstatic hyperplasia. J. Urol. 148:1467-4174 (1992).

51.- Price, D.T., R.J. Lefkowitz, M.G. Caron, and D.A. Schwinn. Alpha$_1$-adrenergic receptor mRNA expression in human tissues. FASEB J. 7:A141 (1993).

What is claimed is:

1. A nucleic acid molecule encoding a human α_{1a} adrenergic receptor, wherein the human α_{1a} adrenergic receptor has an amino acid sequence as shown in Figure 1.

2. A nucleic acid molecule encoding a human α_{1b} adrenergic receptor, wherein the human α_{1b} adrenergic receptor has an amino acid sequence as shown in Figure 2.

3. A nucleic acid molecule encoding a human α_{1c} adrenergic receptor, wherein the human α_{1c} adrenergic receptor has an amino acid sequence as shown in Figure 3.

4. The nucleic acid molecule of claim 1, 2, or 3, wherein the nucleic acid molecule is a DNA molecule.

5. The nucleic acid molecule of claim 5, wherein the DNA molecule in a cDNA molecule.

6. A vector comprising a nucleic acid molecule according to any one of claims 1 to 5.

7. The vector of claim 6 which is a plasmid.

8. The vector of claim 6 or 7 adapted for expression in a bacterial cell which comprises the regulatory elements necessary for expression of said nucleic acid in a bacterial cell so located relative to said nucleic acid encoding a human α_1 adrenergic receptor as to permit expression thereof.

9. The vector of claim 6 or 7 adapted for expression in a yeast cell which comprises the regulatory elements

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necessary for the expression of said nucleic acid in a yeast cell so located relative to said nucleic acid encoding a human α_1 adrenergic receptor as to permit expression thereof.

10. The vector of claim 6 or 7 adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of said nucleic acid in the mammalian cell so located relative to said nucleic acid encoding a human α_1 adrenergic receptor as to permit expression thereof.

11. A plasmid designated pcEXV-α1a (ATCC Accession No. 75319).

12. A plasmid designated pcEXV-α1b (ATCC Accession No. 75318).

13. A plasmid designated pcEXV-α1c (ATCC Accession No. 75317).

14. A mammalian cell comprising a vector of claim 6, 7, or 10, or a plasmid of claim 11, 12, or 13.

15. The mammalian cell of claim 14 which is an LM(tk-) cell.

16. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides identical or complementary to a unique sequence included within the sequence of a nucleic acid molecule encoding a human α_{1a} receptor, wherein the nucleic acid molecule has a nucleic acid sequence as shown in Figure 1.

17. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides identical or complementary to a unique sequence included within

the sequence of a nucleic acid molecule encoding a human α_{1b} receptor, wherein the nucleic acid molecule has a nucleic acid sequence as shown in Figure 2.

18. A nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides identical or complementary to a unique sequence included within the sequence of a nucleic acid molecule encoding a human α_{1c} receptor, wherein the nucleic acid molecule has a nucleic acid sequence as shown in Figure 3.

19. The nucleic acid probe of claim 16, 17 or 18, wherein the nucleic acid is DNA.

20. The nucleic acid probe of claim 19, which comprises degenerate oligonucleotides.

21. An antisense oligonucleotide having a sequence which is complementary to a mRNA molecule encoded by a nucleic acid of claim 1.

22. An antisense oligonucleotide having a sequence which is complementary to a mRNA molecule encoded by a nucleic acid of claim 2.

23. An antisense oligonucleotide having a sequence which is complementary to a mRNA molecule encoded by a nucleic acid of claim 3.

24. An antisense oligonucleotide having a sequence which is complementary to a cDNA molecule of claim 5.

25. Antisense oligonucleotides comprising degenerate oligonucleotides of an antisense oligonucleotide of claim 21, 22, 23, or 24.

26. The antisense oligonucleotide of claim 21, 22, 23,

24, or 25 comprising chemical analogs of nucleotides.

27. A method for detecting expression of a specific human α_1 adrenergic receptor, which comprises obtaining RNA from cells or tissue, contacting the RNA so obtained with a nucleic acid probe of claim 16, 17 or 18 under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe, the presence of mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.

28. A method for detecting expression of a specific human α_1 adrenergic receptor in a cell or tissue by in situ hybridization, which comprises contacting the cell or tissue with a nucleic acid probe of claim 19 or an antisense oligonucleotide of claims 21, 22 or 23 under hybridizing conditions, detecting the presence of any mRNA hybridized to the probe indicating expression of the specific human α_1 adrenergic receptor, and thereby detecting the expression of the specific human α_1 adrenergic receptor.

29. A method of isolating a gene encoding a receptor by nucleic acid sequence homology using a nucleic acid probe of claim 19 or 20.

30. The method of claim 29, which comprises using the polymerase chain reaction to obtain a DNA molecule by nucleic acid sequence homology, the DNA molecule of which is used to isolate a gene encoding a receptor.

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31. A method of isolating a DNA of claim 4, which comprises growing bacteria transformed with a vector comprising the DNA of claim 4, lysing the cells and purifying the DNA from the lysed cells.

32. A human α_{1a} adrenergic receptor protein encoded by a nucleic acid molecule according to claim 1.

33. A human α_{1b} adrenergic receptor protein encoded by a nucleic acid molecule according to claim 2.

34. A human α_{1c} adrenergic receptor protein encoded by a nucleic acid molecule according to claim 3.

35. A method of preparing a human α_{1a} adrenergic receptor protein of claim 32, which comprises inducing cells to express the human α_{1a} adrenergic receptor protein, recovering the human α_{1a} adrenergic receptor from the resulting cells, and purifying the human α_{1a} adrenergic receptor so recovered.

36. A method of preparing a human α_{1b} adrenergic receptor protein of claim 33, which comprises inducing cells to express the human α_{1b} adrenergic receptor protein, recovering the human α_{1b} adrenergic receptor from the resulting cells, and purifying the human α_{1b} adrenergic receptor so recovered.

37. A method of preparing a human α_{1c} adrenergic receptor protein of claim 34, which comprises inducing cells to express the human α_{1c} adrenergic receptor protein, recovering the human α_{1c} adrenergic receptor from the resulting cells, and purifying the human α_{1c} adrenergic receptor so recovered.

38. A method of preparing a human α_{1a} adrenergic receptor of claim 32, which comprises inserting a nucleic

acid molecule encoding the human α_{1a} adrenergic receptor protein in a suitable vector, inserting the resulting vector in a suitable host cell so as to produce a cell which expresses the human α_{1a} adrenergic receptor protein, recovering the human α_{1a} adrenergic receptor produced by the resulting cells, and purifying the human α_{1a} adrenergic receptor so recovered.

39. A method of preparing a human α_{1b} adrenergic receptor of claim 33, which comprises inserting a nucleic acid molecule encoding the human α_{1b} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host cell so as to produce a cell which expresses the human α_{1b} adrenergic receptor, recovering the human α_{1b} adrenergic receptor produced by the resulting cell, and purifying the human α_{1b} adrenergic receptor so recovered.

40. A method of preparing a human α_{1c} adrenergic receptor protein of claim 34, which comprises inserting a nucleic acid molecule encoding the human α_{1c} adrenergic receptor in a suitable vector, inserting the resulting vector in a suitable host cell so as to produce a cell which expresses the human α_{1c} adrenergic receptor, recovering the human α_{1c} adrenergic receptor produced by the resulting cell, and purifying the human α_{1c} adrenergic receptor so recovered.

41. An antibody directed to the human α_{1a} adrenergic receptor of claim 32 or to a protein fragment thereof.

42. An antibody directed to the human α_{1b} adrenergic receptor of claim 33 or to a protein fragment

thereof.

43. An antibody directed to the human α_{1c} adrenergic receptor of claim 34 or to a protein fragment thereof.

44. A pharmaceutical composition comprising an effective amount of an antisense oligonucleotide of claim 21 effective to reduce expression of a human α_{1a} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1a} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

45. A pharmaceutical composition comprising an effective amount of an antisense oligonucleotide of claim 22 effective to reduce expression of a human α_{1b} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1b} adrenergic receptor in the cell so as to prevent its translation and a pharmaceutically acceptable carrier.

46. A pharmaceutical composition comprising an effective amount of an antisense oligonucleotide of claim 23 effective to reduce expression of a human α_{1c} adrenergic receptor by passing through a cell membrane and specifically binding with mRNA encoding a human α_{1c} adrenergic receptor in the cell so as to prevent translation and a pharmaceutically acceptable carrier.

47. The pharmaceutically composition of claims 44, 45, or 46, wherein the antisense oligonucleotide is coupled to a substance which inactivates mRNA.

48. The pharmaceutical composition of claim 47, wherein the substance which inactivates the mRNA is a ribozyme.

49. The pharmaceutical composition of claim 44, 45, 46, 47 or 48, wherein the pharmaceutically acceptable carrier capable of passing through a cell membrane comprises a structure which binds to a transporter specific for a selected cell type and is thereby taken up by the cells of the selected cell type.

50. A pharmaceutical composition which comprises an amount of the antibody of claim 41 effective to block binding of naturally occurring substrates to a human α_{1a} adrenergic receptor and a pharmaceutically acceptable carrier.

51. A pharmaceutical composition which comprises an amount of the antibody of claim 42 effective to block binding of naturally occurring substrates to a human α_{1b} adrenergic receptor and a pharmaceutically acceptable carrier.

52. A pharmaceutical composition which comprises an amount of the antibody of claim 43 effective to block binding of naturally occurring substrates to a human α_{1c} adrenergic receptor and a pharmaceutically acceptable carrier.

53. A method of determining the physiological effects of varying the levels of expression of a specific human α_1 adrenergic receptor which comprises producing a transgenic nonhuman mammal whose levels of expression of a human α_1 adrenergic receptor can be varied by use of an inducible promoter.

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54. A method of determining the physiological effects of

expressing varying levels of a specific human α_1 adrenergic receptor which comprises producing a panel of transgenic nonhuman mammals each expressing a different amount of a human α_1 adrenergic receptor.

55. A process for identifying a chemical compound which specifically binds to a human α_{1a} adrenergic receptor, which comprises contacting nonneuronal cells expressing on their cell surface the human α_{1a} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the chemical compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the human α_{1a} adrenergic receptor.

56. A process for identifying a chemical compound which specifically binds to a human α_{1b} adrenergic receptor, which comprises contacting nonneuronal cells expressing on their cell surface the human α_{1b} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the chemical compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the human α_{1b} adrenergic receptor.

57. A process for identifying a chemical compound which specifically binds to a human α_{1c} adrenergic receptor, which comprises contacting nonneuronal cells expressing on their cell surface the human α_{1c} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the chemical compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the human α_{1c} adrenergic receptor.

58. A process involving competitive binding for identifying a chemical compound which specifically

binds to a human α_{1a} adrenergic receptor, which comprises separately contacting nonneuronal cells expressing on their cell surface the human α_{1a} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the both the chemical compound and a second chemical compound known to bind to the human α_{1a} adrenergic receptor, and with only the second chemical compound, under conditions suitable for binding of both chemical compounds, and detecting specific binding of the chemical compound to the human α_{1a} adrenergic receptor, a decrease in binding of the second chemical compound to the human α_{1a} adrenergic receptor in the presence of the chemical compound indicating that the chemical compound binds to the human α_{1a} adrenergic receptor.

59. A process involving competitive binding for identifying a chemical compound which specifically binds to a human α_{1b} adrenergic receptor, which comprises separately contacting nonneuronal cells expressing on their cell surface the human α_{1b} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the both the chemical compound and a second chemical compound known to bind to the human α_{1b} adrenergic receptor, and with only the second chemical compound, under conditions suitable for binding of both chemical compounds, and detecting specific binding of the chemical compound to the human α_{1b} adrenergic receptor, a decrease in binding of the second chemical compound to the human α_{1b} adrenergic receptor in the presence of the chemical compound indicating that the chemical compound binds to the human α_{1b} adrenergic receptor.

60. A process involving competitive binding for

identifying a chemical compound which specifically binds to a human α_{1c} adrenergic receptor, which comprises separately contacting nonneuronal cells expressing on their cell surface the human α_{1c} adrenergic receptor, or a membrane fraction prepared from a cell extract of such cells, with the both the chemical compound and a second chemical compound known to bind to the human α_{1c} adrenergic receptor, and with only the second chemical compound, under conditions suitable for binding of both chemical compounds, and detecting specific binding of the chemical compound to the human α_{1c} adrenergic receptor, a decrease in binding of the second chemical compound to the human α_{1c} adrenergic receptor in the presence of the chemical compound indicating that the chemical compound binds to the human α_{1c} adrenergic receptor.

61. A process for determining whether a chemical compound specifically binds to and activates a human α_{1a} adrenergic receptor, which comprises contacting nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1a} adrenergic receptor, or a membrane fraction prepared form a cell extract of such cells, with the chemical compound under conditions suitable for activation of the human α_{1a} adrenergic receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in second messenger response in the presence of the chemical compound indicating that the chemical compound activates the human α_{1a} adrenergic receptor.

62. A process for determining whether a chemical compound specifically binds to and activates a human α_{1b} adrenergic receptor, which comprises contacting

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nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1b} adrenergic receptor, or a membrane fraction from a cell extract of such cells, with the chemical compound under conditions suitable for activation of the human α_{1b} adrenergic receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in second messenger response in the presence of the chemical compound indicating that the chemical compound activates the human α_{1b} adrenergic receptor.

63. A process for determining whether a chemical compound specifically binds to and activates a human α_{1c} adrenergic receptor, which comprises contacting nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1c} adrenergic receptor, or a membrane fraction from a cell extract of such cells, with the chemical compound under conditions suitable for activation of the human α_{1c} adrenergic receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in second messenger response in the presence of the chemical compound indicating that the chemical compound activates the human α_{1c} adrenergic receptor.

64. A process for determining whether a chemical compound specifically binds to and inhibits a human α_{1a} adrenergic receptor, which comprises separately contacting nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1a} adrenergic receptor, or a membrane fraction from a cell extract of such cells, with both the chemical compound and a second chemical compound known to activate the human α_{1a} adrenergic receptor, and with only the second

chemical compound, under conditions suitable for activation of the human α_{1a} adrenergic receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits the activation of the human α_{1a} adrenergic receptor.

65. A process for determining whether a chemical compound specifically binds to and inhibits a human α_{1b} adrenergic receptor, which comprises separately contacting nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1b} adrenergic receptor, or a membrane fraction from a cell extract of such cells, with both the chemical compound and a second chemical compound known to activate the human α_{1b} adrenergic receptor, and with only the second chemical compound, under conditions suitable for activation of the human α_{1b} adrenergic receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits the activation of the human α_{1b} adrenergic receptor.

66. A process for determining whether a chemical

compound specifically binds to and inhibits a human α_{1c} adrenergic receptor, which comprises separately contacting nonneuronal cells producing a second messenger response and expressing on their cell surface a human α_{1c} adrenergic receptor, or a membrane fraction from a cell extract of such cells, with both the chemical compound and a second chemical compound known to activate the human α_{1c} adrenergic receptor, and with only the second chemical compound, under conditions suitable for activation of the human α_{1c} adrenergic receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits the activation of the human α_{1c} adrenergic receptor.

67. The process of claim 61, 62, or 63, wherein the second messenger response comprises activation of phosphatidyl inositol specific phospholipase C and the change in second messenger response is an increase in phosphatidyl inositol lipid metabolism.

68. The process of claim 61, 62, or 63, wherein the second messenger response comprises intracellular calcium levels and the change in second messenger response is an increase in intracellular calcium levels.

69. The process of claim 64, 65, or 66, wherein the second messenger response is activation of phosphatidyl inositol specific phospholipase C and

the change in second messenger response is a smaller increase in phosphatidyl inositol lipid metabolism in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

70. The process of claim 64, 65, or 66, wherein the second messenger response comprises intracellular calcium levels and the change in second messenger response is a smaller increase in the level of intracellular calcium in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

71. A method for detecting the presence of a human α_{1a} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody of claim 41, under conditions that permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby detecting the presence of a human α_{1a} adrenergic receptor on the surface of the cell.

72. A method for detecting the presence of a human α_{1b} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody of claim 42, under conditions that permit binding of the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby detecting the presence of a human α_{1b} adrenergic receptor on the surface of the cell.

73. A method for detecting the presence of a human α_{1c} adrenergic receptor on the surface of a cell, which comprises contacting the cell with an antibody of claim 43, under conditions that permit binding of

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the antibody to the receptor, detecting the presence of any of the antibody bound to the cell, and thereby detecting the presence of a human α_{1c} adrenergic receptor on the surface of the cell.

74. A method for diagnosing a predisposition to a disorder or a disorder associated with the expression of a specific human α_1 adrenergic receptor allele which comprises:

(a) obtaining DNA from subjects suffering from a disorder;

(b) performing a restriction digest of the DNA with a panel of restriction enzymes;

(c) electrophoretically separating the resulting DNA fragments on a sizing gel;

(d) contacting the gel with a nucleic acid probe of claim 16, 17, or 18 labelled with a detectable marker;

(e) detecting the labelled bands which have hybridized to the DNA encoding either an α_{1a}, α_{1b} or α_{1c} adrenergic receptor, labelled with the detectable marker to create a unique band pattern specific to the DNA of subjects suffering with the disorder;

(f) preparing DNA for diagnosis by steps (a) to (e);

(g) comparing the unique band pattern specific to the DNA of patients suffering from the disorder from step (e) and DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose

thereby predisposition to the disorder if the patterns are the same.

75. The method of Claim 74, wherein a disorder associated with the expression of a specific human α_{1a}, α_{1b}, or α_{1c} allele is diagnosed.

76. Use of a transgenic nonhuman mammal for identifying a substance capable of alleviating the abnormalities resulting from overexpression of a specific human α_1 adrenergic receptor, by determining if a substance administered to the transgenic nonhuman mammal alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of overexpression of the human α_1 adrenergic receptor subtype.

77. Use of a transgenic nonhuman mammal for identifying a substance capable of alleviating the abnormalities resulting from underexpression of a human α_1 adrenergic receptor subtype, by determining if a substance administered to the transgenic nonhuman mammal alleviates the physical and behavioral abnormalities displayed by the transgenic nonhuman mammal as a result of underexpression of the human α_1 adrenergic receptor subtype.

78. A membrane fraction isolated from a cell of Claim 14.

79. A method of preparing a composition which comprises identifying a compound using the process of any one of Claims 55 to 70 and admixing the compound so identified with a carrier.

80. The method of claim 79 wherein the carrier is a pharmaceutically acceptable carrier.

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FIGURE 1A, 1/37
FIGURE 1B, 2/37
FIGURE 1C, 3/37
FIGURE 1D, 4/37
FIGURE 1E, 5/37
FIGURE 1F, 6/37
FIGURE 1G, 7/37
FIGURE 1H, 8/37
FIGURE 1I, 9/37

FIGURE 1A


```
                -170                -150                -130
                  .                   .                   .                   .                   .
-176  CCGGGCCAGGCACGTCCGCTCTCGGACAGCCGCTCCGCGTCACAGGAACTTGGGCAGGAC  -117

                -110                -90                 -70
                  .                   .                   .                   .                   .
-116  CCGACGGGACCCGTGCGCGGAGCTGCATCTGGAGCCCCGCGGCTATGCCCTGTGCTCCCC  -57

                -50                 -30                 -10
                  .                   .                   .                   .                   .
 -56  TCCTGCCGGCCGCTCGTTCTGTGCCCCCGGCCCGGCCACCGACGGCCGCGCGTTGAGATG  3
 -18                                                                M  1

                 10                  30                  50
                  .                   .                   .                   .                   .
   4  ACTTTCCGCGATCTCCTGAGCGTCAGTTTCGAGGGACCCCGCCCGGACAGCAGCGCAGGG  63
   2  T  F  R  D  L  L  S  V  S  F  E  G  P  R  P  D  S  S  A  G    21
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FIGURE 1B

```
                70                  90                  110
                .                   .                   .
64   GGCTCCAGCGCGGGCGGCGGCGGGGGCAGCGCGGGCGGCGCGGCCCCCTCGGAGGGCCCG   123
22   G  S  S  A  G  G  G  G  G  S  A  G  G  A  A  P  S  E  G  P     41

          130                 150                 170
          .                   .                   .
124  GCGGTGGGCGGCGTGCCGGGGGGCGCGGGCGGCGGCGGCGGCGTGGTGGGCGCAGGCAGC   183
42   A  V  G  G  V  P  G  G  A  G  G  G  G  V  V  G  A  G  S     61

          190                 210                 230
          .                   .                   .
184  GGCGAGGACAACCGGAGCTCCGCGGGGGAGCCGGGGAGCGCGGGCGCGGGCGGCGACGTG   243
62   G  E  D  N  R  S  S  A  G  E  P  G  S  A  G  A  G  G  D  V     81

          250                 270                 290
          .                   .                   .
244  AATGGCACGGCGGCCGTCGGGGGACTGGTGGTGAGCGCGCAGGGCGTGGGCGTGGGCGTC   303
82   N  G  T  A  A  V  G  G  L  V  V  S  A  Q  G  V  G  V  G  V     101
```

FIGURE 1C

```
          310                 330                 350
304  TTCCTGGCAGCCTTCATCCTTATGGCCGTGGCAGGTAACCTGCTTGTCATCCTCTCAGTG  363
102  F  L  A  A  F  I  L  M  A  V  A  G  N  L  L  V  I  L  S  V  121

          370                 390                 410
364  GCCTGCAACCGCCACCTGCAGACCGTCACCAACTATTTCATCGTGAACCTGGCCGTGGCC  423
122  A  C  N  R  H  L  Q  T  V  T  N  Y  F  I  V  N  L  A  V  A  141

          430                 450                 470
424  GACCTGCTGCTGAGCGCCACCGTACTGCCCTTCTCGGCCACCATGGAGGTTCTGGGCTTC  483
142  D  L  L  L  S  A  T  V  L  P  F  S  A  T  M  E  V  L  G  F  161

          490                 510                 530
484  TGGGCCTTTGGCCGCGCCTTCTGCGACGTATGGGCCGCCGTGGACGTGCTGTGCTGCACG  543
162  W  A  F  G  R  A  F  C  D  V  W  A  A  V  D  V  L  C  C  T  181
```

FIGURE 1D

```
          550                 570                 590
           .                   .                   .                   .                   .
544  GCCTCCATCCTCAGCCTCTGCACCATCTCCGTGGACCGGTACGTGGGCGTGCGCCACTCA  603
182  A  S  I  L  S  L  C  T  I  S  V  D  R  Y  V  G  V  R  H  S   201

          610                 630                 650
           .                   .                   .                   .                   .
604  CTCAAGTACCCAGCCATCATGACCGAGCGCAAGGCGGCCGCCATCCTGGCCCTGCTCTGG  663
202  L  K  Y  P  A  I  M  T  E  R  K  A  A  A  I  L  A  L  L  W   221

          670                 690                 710
           .                   .                   .                   .                   .
664  GTCGTAGCCCTGGTGGTGTCCGTAGGGCCCCTGCTGGGCTGGAAGGAGCCCGTGCCCCCT  723
222  V  V  A  L  V  V  S  V  G  P  L  L  G  W  K  E  P  V  P  P   241

          730                 750                 770
           .                   .                   .                   .                   .
724  GACGAGCGCTTCTGCGGTATCACCGAGGAGGCGGGCTACGCTGTCTTCTCCTCCGTGTGC  783
242  D  E  R  F  C  G  I  T  E  E  A  G  Y  A  V  F  S  S  V  C   261
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FIGURE 1E

```
          790                       810                       830
           .                         .                         .                         .                         .
784  TCCTTCTACCTGCCCATGGCGGTCATCGTGGTCATGTACTGCCGCGTGTACGTGGTCGCG  843
262  S  F  Y  L  P  M  A  V  I  V  V  M  Y  C  R  V  Y  V  V  A    281

          850                       870                       890
           .                         .                         .                         .                         .
844  CGCAGCACCACGCGCAGCCTCGAGGCAGGCGTCAAGCGCGAGCGAGGCAAGGCCTCCGAG  903
282  R  S  T  T  R  S  L  E  A  G  V  K  R  E  R  G  K  A  S  E    301

          910                       930                       950
           .                         .                         .                         .                         .
904  GTGGTGCTGCGCATCCACTGTCGCGGCGCGGCCACGGGCGCCGACGGGGCGCACGGCATG  963
302  V  V  L  R  I  H  C  R  G  A  A  T  G  A  D  G  A  H  G  M    321

          970                       990                      1010
           .                         .                         .                         .                         .
964  CGCAGCGCCAAGGGCCACACCTTCCGCAGCTCGCTCTCCGTGCGCCTGCTCAAGTTCTCC  1023
322  R  S  A  K  G  H  T  F  R  S  S  L  S  V  R  L  L  K  F  S    341
```

FIGURE 1F

```
          1030                1050                1070
           .                   .                   .                   .
1024  CGTGAGAAGAAAGCGGCCAAGACTCTGGCCATCGTCGTGGGTGTCTTCGTGCTCTGCTGG  1083
 342  R  E  K  K  A  A  K  T  L  A  I  V  V  G  V  F  V  L  C  W     361

          1090                1110                1130
           .                   .                   .                   .
1084  TTCCCTTTCTTCTTTGTCCTGCCGCTCGGCTCCTTGTTCCCGCAGCTGAAGCCATCGGAG  1143
 362  F  P  F  F  F  V  L  P  L  G  S  L  F  P  Q  L  K  P  S  E     381

          1150                1170                1190
           .                   .                   .                   .
1144  GGCGTCTTCAAGGTCATCTTCTGGCTCGGCTACTTCAACAGCTGCGTGAACCCGCTCATC  1203
 382  G  V  F  K  V  I  F  W  L  G  Y  F  N  S  C  V  N  P  L  I     401

          1210                1230                1250
           .                   .                   .                   .
1204  TACCCCTGTTCCAGCCGCGAGTTCAAGCGCGCCTTCCTCCGTCTCCTGCGCTGCCAGTGC  1263
 402  Y  P  C  S  S  R  E  F  K  R  A  F  L  R  L  L  R  C  Q  C     421
```

FIGURE 1G

```
          1270                1290                1310
            .                   .                   .                   .
1264  CGTCGTCGCCGGCGCCGCCGCCCTCTCTGGCGTGTCTACGGCCACCACTGGCGGGCCTCC  1323
 422  R  R  R  R  R  R  R  P  L  W  R  V  Y  G  H  H  W  R  A  S    441

          1330                1350                1370
            .                   .                   .                   .
1324  ACCAGCGGCCTGCGCCAGGACTGCGCCCCGAGTTCGGGCGACGCGCCCCCCGGAGCGCCG  1383
 442  T  S  G  L  R  Q  D  C  A  P  S  S  G  D  A  P  P  G  A  P    461

          1390                1410                1430
            .                   .                   .                   .
1384  CTGGCCCTCACCGCGCTCCCCGACCCCGACCCCGAACCCCCAGGCACGCCCGAGATGCAG  1443
 462  L  A  L  T  A  L  P  D  P  D  P  E  P  P  G  T  P  E  M  Q    481

          1450                1470                1490
            .                   .                   .                   .
1444  GCTCCGGTCGCCAGCCGTCGAAAGCCACCCAGCGCCTTCCGCGAGTGGAGGCTGCTGGGG  1503
 482  A  P  V  A  S  R  R  K  P  P  S  A  F  R  E  W  R  L  L  G    501
```

FIGURE 1H

```
          1510                1530                1550
           .                   .                   .                   .
1504  CCGTTCCGGAGACCCACGACCCAGCTGCGCGCCAAAGTCTCCAGCCTGTCGCACAAGATC  1563
 502  P  F  R  R  P  T  T  Q  L  R  A  K  V  S  S  L  S  H  K  I    521

          1570                1590                1610
           .                   .                   .                   .
1564  CGCGCCGGGGGCGCGCAGCGCGCAGAGGCAGCGTGCGCCCAGCGCTCAGAGGTGGAGGCT  1623
 522  R  A  G  G  A  Q  R  A  E  A  A  C  A  Q  R  S  E  V  E  A    541

          1630                1650                1670
           .                   .                   .                   .
1624  GTGTCCCTAGGCGTCCCACACGAGGTGGCCGAGGGCGCCACCTGCCAGGCCTACGAATTG  1683
 542  V  S  L  G  V  P  H  E  V  A  E  G  A  T  C  Q  A  Y  E  L    561

          1690                1710                1730
           .                   .                   .                   .
1684  GCCGACTACAGCAACCTACGGGAGACCGATATTTAAGGACCCCAGAGCTAGGCCGCGGAG  1743
 562  A  D  Y  S  N  L  R  E  T  D  I  *                            572
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FIGURE 11

```
      1750                1770                1790
       .                   .                   .                   .                   .                   .
1744  TGTGCTGGGCTTGGGGGTAAGGGGGACCAGAGAGGCGGGCTGGTGTTCTAAGAGCCCCCG  1803

      1810                1830                1850
       .                   .                   .                   .                   .                   .
1804  TGCAAATCGGAGACCCGGAAACTGATCAGGGCAGCTGCTCTGTGACATCCCTGAGGAACT  1863

      1870                1890                1910
       .                   .                   .                   .                   .                   .
1864  GGGCAGAGCTTGAGGCTGGAGCCCTTGAAAGGTGAAAAGTAGTGGGGCCCCCTGCTGGAC  1923

      1930                1950
       .                   .                   .                   .
1924  TCAGGTGCCCAGAACTCTTTTCTTAGAAGGGAGAGGCTGC  1963
```

FIGURE 2A, 10/37
FIGURE 2B, 11/37
FIGURE 2C, 12/37
FIGURE 2D, 13/37
FIGURE 2E, 14/37
FIGURE 2F, 15/37
FIGURE 2G, 16/37
FIGURE 2H, 17/37

FIGURE 2A

```
          120                -100                -80
          .                  .                   .                   .
-122 GCCAGGAGGGCGCCTCTGGGAAGAAGACCACGGGGGAAGCAAAGTTTCAGGGCAGCTGAG -63
      -60                -40                 -20
      .                  .                   .                   .
 -62 GAGCCTTCGCCGCAGCCCTTCCGAGCCCAATCATCCCCCAGGCTATGGAGGGCGGACTCT  -3
       0                  20                  40
       .                  .                   .                   .
  -2 AAGATGAATCCCGACCTGGACACCGGCCACAACACATCAGCACCTGCCCACTGGGGAGAG  57
   0    M   N   P   D   L   D   T   G   H   N   T   S   A   P   A   H   W   G   E   19

      60                 80                  100
      .                  .                   .                   .
  58 TTGAAAAATGCCAACTTCACTGGCCCCAACCAGACCTCGAGCAACTCCACACTGCCCCAG 117
  20 L   K   N   A   N   F   T   G   P   N   Q   T   S   S   N   S   T   L   P   Q   39
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FIGURE 2B

```
        120                 140                 160
         .                   .                   .                   .                   .
118     CTGGACATCACCAGGGCCATCTCTGTGGGCCTGGTGCTGGGCGCCTTCATCCTCTTTGCC   177
 40     L  D  I  T  R  A  I  S  V  G  L  V  L  G  A  F  I  L  F  A     59

        180                 200                 220
         .                   .                   .                   .                   .
178     ATCGTGGGCAACATCCTAGTCATCTTGTCTGTGGCCTGCAACCGGCACCTGCGGACGCCC   237
 60     I  V  G  N  I  L  V  I  L  S  V  A  C  N  R  H  L  R  T  P     79

        240                 260                 280
         .                   .                   .                   .                   .
238     ACCAACTACTTCATTGTCAACCTGGCCATGGCCGACCTGCTGTTGAGCTTCACCGTCCTG   297
 80     T  N  Y  F  I  V  N  L  A  M  A  D  L  L  L  S  F  T  V  L     99

        300                 320                 340
         .                   .                   .                   .                   .
298     CCCTTCTCAGCGGCCCTAGAGGTGCTCGGCTACTGGGTGCTGGGGCGGATCTTCTGTGAC   357
100     P  F  S  A  A  L  E  V  L  G  Y  W  V  L  G  R  I  F  C  D     119
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FIGURE 2C

```
      360                 380                 400
       .         .         .         .         .         .
358   ATCTGGGCAGCCGTGGATGTCCTGTGCTGCACAGCGTCCATTCTGAGCCTGTGCGCCATC   417
120   I  W  A  A  V  D  V  L  C  C  T  A  S  I  L  S  L  C  A  I    139

      420                 440                 460
       .         .         .         .         .         .
418   TCCATCGATCGCTACATCGGGGTGCGCTACTCTCTGCAGTATCCCACGCTGGTCACCCGG   477
140   S  I  D  R  Y  I  G  V  R  Y  S  L  Q  Y  P  T  L  V  T  R    159

      480                 500                 520
       .         .         .         .         .         .
478   AGGAAGGCCATCTTGGCGCTGCTCAGTGTCTGGGTCTTGTCCACCGTCATCTCCATCGGG   537
160   R  K  A  I  L  A  L  L  S  V  W  V  L  S  T  V  I  S  I  G    179

      540                 560                 580
       .         .         .         .         .         .
538   CCTCTCCTTGGGTGGAAGGAGCCGGCACCCAACGATGACAAGGAGTGCGGGGTCACCGAA   597
180   P  L  L  G  W  K  E  P  A  P  N  D  D  K  E  C  G  V  T  E    199
```

FIGURE 2D

```
        600                 620                 640
         .                   .                   .                   .                   .
598     GAACCCTTCTATGCCCTCTTCTCCTCTCTGGGCTCCTTCTACATCCCTCTGGCGGTCATT    657
200     E  P  F  F  Y  A  L  F  S  S  L  G  S  F  Y  I  P  L  A  V  I    219

        660                 680                 700
         .                   .                   .                   .                   .
658     CTAGTCATGTACTGCCGTGTCTATATAGTGGCCAAGAGAACCACCAAGAACCTAGAGGCA    717
220     L  V  M  Y  C  R  V  Y  I  V  A  K  R  T  T  K  N  L  E  A    239

        720                 740                 760
         .                   .                   .                   .                   .
718     GGAGTCATGAAGGAGATGTCCAACTCCAAGGAGCTGACCCTGAGGATCCATTCCAAGAAC    777
240     G  V  M  K  E  M  S  N  S  K  E  L  T  L  R  I  H  S  K  N    259

        780                 800                 820
         .                   .                   .                   .                   .
778     TTTCACGAGGACACCCTTAGCAGTACCAAGGCCAAGGGCCACAACCCCAGGAGTTCCATA    837
260     F  H  E  D  T  L  S  S  T  K  A  K  G  H  N  P  R  S  S  I    279
```

FIGURE 2E

```
          840                 860                 880
           .                   .                   .                   .                   .
838  GCTGTCAAACTTTTTAAGTTCTCCAGGGAAAAGAAAGCAGCTAAGACGTTGGGCATTGTG  897
280  A  V  K  L  F  K  F  S  R  E  K  K  A  A  K  T  L  G  I  V    299

          900                 920                 940
           .                   .                   .                   .                   .
898  GTCGGTATGTTCATCTTGTGCTGGCTACCCTTCTTCATCGCTCTACCGCTTGGCTCCTTG  957
300  V  G  M  F  I  L  C  W  L  P  F  F  I  A  L  P  L  G  S  L    319

          960                 980                 1000
           .                   .                   .                   .                   .
958  TTCTCCACCCTGAAGCCCCCCGACGCCGTGTTCAAGGTGGTGTTCTGGCTGGGCTACTTC  1017
320  F  S  T  L  K  P  P  D  A  V  F  K  V  V  F  W  L  G  Y  F    339

         1020                1040                1060
           .                   .                   .                   .                   .
1018 AACAGCTGCCTCAACCCCATCATCTACCCATGCTCCAGCAAGGAGTTCAAGCGCGCTTTC  1077
340  N  S  C  L  N  P  I  I  Y  P  C  S  S  K  E  F  K  R  A  F    359
```

FIGURE 2F

```
          1080                1100                1120
          .                   .                   .
1078  GTGCGCATCCTCGGGTGCCAGTGCCGCGGCCGCGGCCGCCGCCGACGCCGCCGCCGCCGT  1137
 360  V  R  I  L  G  G  C  Q  C  R  G  R  G  R  R  R  R  R  R  R  R  379

          1140                1160                1180
          .                   .                   .
1138  CGCCTGGGCGGCTGCGCCTACACCTACCGGCCGTGGACGCGCGGCGGCTCGCTGGAGCGC  1197
 380  R  L  G  G  C  A  Y  T  Y  R  P  W  T  R  G  G  S  L  E  R  399

          1200                1220                1240
          .                   .                   .
1198  TCGCAGTCGCGCAAGGACTCGCTGGACGACAGCGGCAGCTGCCTGAGCGGCAGCCAGCGG  1257
 400  S  Q  S  R  K  D  S  L  D  D  S  G  S  C  L  S  G  S  Q  R  419

          1260                1280                1300
          .                   .                   .
1258  ACCCTGCCCTCGGCCTCGCCGAGCCCGGGCTACCTGGGCCGCGGCGCGCCACCGCCAGTC  1317
 420  T  L  P  S  A  S  P  S  P  G  Y  L  G  R  G  A  P  P  P  V  439
```

FIGURE 2G

```
      1320                1340                1360
        .                   .                   .                   .
1318  GAGCTGTGCGCCTTCCCCGAGTGGAAGGCGCCCGGCGCCCTCCTGAGCCTGCCCGCGCCT  1377
 440  E  L  C  A  F  P  E  W  K  A  P  G  A  L  L  S  L  P  A  P    459

      1380                1400                1420
        .                   .                   .                   .
1378  GAGCCCCCCGGCCGCCGCGGCCGCCACGACTCGGGCCCGCTCTTCACCTTCAAGCTCCTG  1437
 460  E  P  P  G  R  R  G  R  H  D  S  G  P  L  F  T  F  K  L  L    479

      1440                1460                1480
        .                   .                   .                   .
1438  ACCGAGCCCGAGAGCCCCGGGACCGACGGCGGCGCCAGCAACGGAGGCTGCGAGGCCGCG  1497
 480  T  E  P  E  S  P  G  T  D  G  G  A  S  N  G  G  C  E  A  A    499

      1500                1520                1540
        .                   .                   .                   .
1498  GCCGACGTGGCCAACGGGCAGCCGGGCTTCAAAAGCAACATGCCCCTGGCGCCCGGGCAG  1557
 500  A  D  V  A  N  G  Q  P  G  F  K  S  N  M  P  L  A  P  G  Q    519
```

FIGURE 2H

```
                1560                1580                1600
                 .                   .                   .
1558  TTTTAGGGCCCCCGTGCGCAGCTTTCTTTCCCTGGGGAGGAAAACATCGTGGGGGGGA  1615
 520  F  *                                                        520
```

FIGURE 3A, 18/37
FIGURE 3B, 19/37
FIGURE 3C, 20/37
FIGURE 3D, 21/37
FIGURE 3E, 22/37
FIGURE 3F, 23/37
FIGURE 3G, 24/37

FIGURE 3A

```
                -120                -100                -80
                 .                   .                   .                   .
-124  CCAGCCAAACCACTGGCAGGCTCCCTCCAGCCGAGACCTTTTATTCCCGGCTCCCGAGCT  -65

                -60                 -40                 -20
                 .                   .                   .                   .
 -64  CCGCCTCCGCGCCAGCCCGGGAGGTGGCCCTGACAGCCGGACCTCGCCCGGCCCCGGCTG   -5

                 0                   20                  40
                 .                   .                   .                   .
  -4  GGACCATGGTGTTTCTCTCGGGAAATGCTTCCGACAGCTCCAACTGCACCCAACCGCCGG   55
   0      M  V  F  L  S  G  N  A  S  D  S  S  N  C  T  Q  P  P  A   19

                60                  80                  100
                 .                   .                   .                   .
  56  CACCGGTGAACATTTCCAAGGCCATTCTGCTCGGGGTGATCTTGGGGGGCCTCATTCTTT   115
  20    P  V  N  I  S  K  A  I  L  L  G  V  I  L  G  G  L  I  L  F   39
```

FIGURE 3B

```
        120                 140                 160
116 TCGGGGTGCTGGGTAACATCCTAGTGATCCTCTCCGTAGCCTGTCACCGACACCTGCACT 175
 40   G  V  L  G  N  I  L  V  I  L  S  V  A  C  H  R  H  L  H  S  59

        180                 200                 220
176 CAGTCACGCACTACTACATCGTCAACCTGGCGGTGGCCGACCTCCTGCTCACCTCCACGG 235
 60   V  T  H  Y  Y  I  V  N  L  A  V  A  D  L  L  L  T  S  T  V  79

        240                 260                 280
236 TGCTGCCCTTCTCCGCCATCTTCGAGGTCCTAGGCTACTGGGCCTTCGGCAGGGTCTTCT 295
 80   L  P  F  S  A  I  F  E  V  L  G  Y  W  A  F  G  R  V  F  C  99

        300                 320                 340
296 GCAACATCTGGGCGGCAGTGGATGTGCTGTGCTGCACCGCGTCCATCATGGGCCTCTGCA 355
100   N  I  W  A  A  V  D  V  L  C  C  T  A  S  I  M  G  L  C  I  119
```

FIGURE 3C

```
          360                 380                 400
356  TCATCTCCATCGACCGCTACATCGGCGTGAGCTACCCGCTGCGCTACCCAACCATCGTCA   415
120    I  S  I  D  R  Y  I  G  V  S  Y  P  L  R  Y  P  T  I  V  T   139

          420                 440                 460
416  CCCAGAGGAGGGGTCTCATGGCTCTGCTCTGCGTCTGGGCACTCTCCCTGGTCATATCCA   475
140    Q  R  R  G  L  M  A  L  L  C  V  W  A  L  S  L  V  I  S  I   159

          480                 500                 520
476  TTGGACCCCTGTTCGGCTGGAGGCAGCCGGCCCCCGAGGACGAGACCATCTGCCAGATCA   535
160    G  P  L  F  G  W  R  Q  P  A  P  E  D  E  T  I  C  Q  I  N   179

          540                 560                 580
536  ACGAGGAGCCGGGCTACGTGCTCTTCTCAGCGCTGGGCTCCTTCTACCTGCCTCTGGCCA   595
180    E  E  P  G  Y  V  L  F  S  A  L  G  S  F  Y  L  P  L  A  I   199
```

FIGURE 3D

```
          600                 620                 640
596  TCATCCTGGTCATGTACTGCCGCGTCTACGTGGTGGCCAAGAGGGAGAGCCGGGGCCTCA  655
200    I  L  V  M  Y  C  R  V  Y  V  V  A  K  R  E  S  R  G  L  K  219

          660                 680                 700
656  AGTCTGGCCTCAAGACCGACAAGTCGGACTCGGAGCAAGTGACGCTCCGCATCCATCGGA  715
220    S  G  L  K  T  D  K  S  D  S  E  Q  V  T  L  R  I  H  R  K  239

          720                 740                 760
716  AAAACGCCCCGGCAGGAGGCAGCGGGATGGCCAGCGCCAAGACCAAGACGCACTTCTCAG  775
240    N  A  P  A  G  G  S  G  M  A  S  A  K  T  K  T  H  F  S  V  259

          780                 800                 820
776  TGAGGCTCCTCAAGTTCTCCCGGGAGAAGAAAGCGGCCAAAACGCTGGGCATCGTGGTCG  835
260    R  L  L  K  F  S  R  E  K  K  A  A  K  T  L  G  I  V  V  G  279
```

FIGURE 3E

```
          840                 860                 880
836 GCTGCTTCGTCCTCTGCTGGCTGCCTTTTTTCTTAGTCATGCCCATTGGGTCTTTCTTCC 895
280   C  F  V  L  C  W  L  P  F  F  L  V  M  P  I  G  S  F  F  P 299

          900                 920                 940
896 CTGATTTCAAGCCCTCTGAAACAGTTTTTAAAATAGTATTTTGGCTCGGATATCTAAACA 955
300   D  F  K  P  S  E  T  V  F  K  I  V  F  W  L  G  Y  L  N  S 319

          960                 980                 1000
956 GCTGCATCAACCCCATCATATACCCATGCTCCAGCCAAGAGTTCAAAAAGGCCTTTCAGA 1015
320   C  I  N  P  I  I  Y  P  C  S  S  Q  E  F  K  K  A  F  Q  N 339

          1020                1040                1060
1016 ATGTCTTGAGAATCCAGTGTCTCTGCAGAAAGCAGTCTTCCAAACATGCCCTGGGCTACA 1075
340   V  L  R  I  Q  C  L  C  R  K  Q  S  S  K  H  A  L  G  Y  T 359
```

FIGURE 3F

```
          1080                1100                1120
1076  CCCTGCACCCGCCCAGCCAGGCCGTGGAAGGGCAACACAAGGACATGGTGCGCATCCCCG  1135
 360    L  H  P  P  S  Q  A  V  E  G  Q  H  K  D  M  V  R  I  P  V   379

          1140                1160                1180
1136  TGGGATCAAGAGAGACCTTCTACAGGATCTCCAAGACGGATGGCGTTTGTGAATGGAAAT  1195
 380    G  S  R  E  T  F  Y  R  I  S  K  T  D  G  V  C  E  W  K  F   399

          1200                1220                1240
1196  TTTTCTCTTCCATGCCCCGTGGATCTGCCAGGATTACAGTGTCCAAAGACCAATCCTCCT  1255
 400    F  S  S  M  P  R  G  S  A  R  I  T  V  S  K  D  Q  S  S  C   419

          1260                1280                1300
1256  GTACCACAGCCCGGGTGAGAAGTAAAAGCTTTTTGCAGGTCTGCTGCTGTGTAGGGCCCT  1315
 420    T  T  A  R  V  R  S  K  S  F  L  Q  V  C  C  C  V  G  P  S   439
```

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FIGURE 3G

```
                1320                1340                1360
                 .                   .                   .
1316  CAACCCCCAGCCTTGACAAGAACCATCAAGTTCCAACCATTAAGGTCCACACCATCTCCC  1375
 440    T   P   S   L   D   K   N   H   Q   V   P   T   I   K   V   H   T   I   S   L   459

                1380                1400                1420
                 .                   .                   .
1376  TCAGTGAGAACGGGGAGGAAGTCTAGGACAGGAAAGATGCAGAGGAAAGGGGAATATCTT  1435
 460    S   E   N   G   E   E   V   *                                                   466

                1440                1460                1480
                 .                   .                   .
1436  AGGTACCATACCCTGGAGTTCTAGAGGATTCCTCGACAAGCTTATTCCGATCCAGACATG  1495

                1500
                 .
1496  ATAGATACATTGATGAGTT  1514
```

FIGURE 4A, 25/37
FIGURE 4B, 26/37
FIGURE 4C, 27/37
FIGURE 4D, 28/37

FIGURE 4A

```
                    1                                                  40
human   alpha1a     mtfrdllsvs fegprpdssa ggssaggggg saggaapseg
H318/3  alpha1a     .......... .......... .......... ..........
Rat     alpha1a     mtfrdilsvt fegprssssst ggsgagggag tvg....peg
        Consensus   MTFRD-LS-- FEGPR--SS- GGS-AGGG-G --G-----EG

                    41                                                 80
human   alpha1a     pavggvpgg- ggggg-vga- sgednrssa. .....gepgs
H318/3  alpha1a     ........m- aalrs-mma- ylsewrtpty rstemvqrlr
Rat     alpha1a     gavggvpg.- tggga-vgt- sgednqsst. ......gepg
        Consensus   ---------A -----V---G ---------- ----------

                    81                                                 120
human   alpha1a     ag-ggdvngt ---------- ---------- ----m-----
H318/3  alpha1a     me-vqhstst ---------- ---------- ----m-----
Rat     alpha1a     aa-sgevngs ---------- ---------- ----t-----
        Consensus   --A------- AAVGGLVVSA QGVGVGVFLA AFIL-AVAGN

                    121                                                160
human   alpha1a     ---------- ---------- ---------- ---t------
H318/3  alpha1a     ---------- ---------- ---------- ---t------
Rat     alpha1a     ---------- ---------- ---------- ---a------
        Consensus   LLVILSVACN RHLQTVTNYF IVNLAVADLL LSA-VLPFSA
```

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FIGURE 4B

```
                    161                                                 200
human   alpha1a     ---------- --a------- ---------- ----------
H318/3  alpha1a     ---------- --a------- ---------- ----------
Rat     alpha1a     ---------- --t------- ---------- ----------
        Consensus   TMEVLGFWAF GR-FCDVWAA VDVLCCTASI LSLCTISVDR

                    201                                                 240
human   alpha1a     ---------- ---------- -------v-- ----------
H318/3  alpha1a     ---------- ---------- -------v-- ----------
Rat     alpha1a     ---------- ---------- -------a-- ----------
        Consensus   YVGVRHSLKY PAIMTERKAA AILALLW-VA LVVSVGPLLG

                    241                                                 280
human   alpha1a     ---------- -------a-- -v-------- ----------
H318/3  alpha1a     ---------- -------a-- -v-------- ----------
Rat     alpha1a     ---------- -------v-- -i-------- ----------
        Consensus   WKEPVPPDER FCGITEE-GY A-FSSVCSFY LPMAVIVVMY

                    281                                                 320
human   alpha1a     ---------- -------v-- -r--------- ----------g
H318/3  alpha1a     ---------- -------v-- -r--------- ----------g
Rat     alpha1a     ---------- -------i-- -p--------- ----------s
        Consensus   CRVYVVARST TRSLEAG-KR E-GKASEVVL RIHCRGAAT-
```

FIGURE 4C

```
                    321                                                 360
human   alpha1a     -d-ah-mr-a ----f----- ---------- ----------
H318/3  alpha1a     -d-ah-mr-a ----f----- ---------- ----------
Rat     alpha1a     -k-yp-tq-s ----l----- ---------- ----------
        Consensus   A-G--G--S- KGHT-RSSLS VRLLKFSREK KAAKTLAIVV

                    361                                                 400
human   alpha1a     ---------- ---------- ---------- ----------
H318/3  alpha1a     ---------- ---------- ---------- ----------
Rat     alpha1a     ---------- ---------- ---------- ----------
        Consensus   GVFVLCWFPF FFVLPLGSLF PQLKPSEGVF KVIFWLGYFN

                    401                                                 440
human   alpha1a     ---------- ---------- ---------- ---.rp-wrv
H318/3  alpha1a     ---------- ---------- ---------- ---.rp-wrv
Rat     alpha1a     ---------- ---------- ---------- ---lws-rpp
        Consensus   SCVNPLIYPC SSREFKRAFL RLLRCQCRRR RRR---L---

                    441                                                 480
human   alpha1a     yg..hhw--. ...stsgl-q dca---gdap --ap-alt-l
H318/3  alpha1a     yg..hhw--. ...stsgl-q dca---gdap --ap-alt-l
Rat     alpha1a     lasldrr--f rlrpqpsh-s prg---phct --cg-grh-.
        Consensus   -------RA- --------R- ---PSS---- PG--L---A-
```

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FIGURE 4D

```
                    481                                                 520
human   alpha1a     pdpdpeppgt pem-apv--r -k..ppsafr ewrllgpfr-
H318/3  alpha1a     pdpdpeppgt pem-apv--r -shpapsasg gcwgrsgdp-
Rat     alpha1a     ......gdag fgl-qsk--l -.......lr ewrllgplq-
        Consensus   ---------- ---Q---AS- R--------- ---------R

                    521                                                 560
human   alpha1a     -ttqlrakvs slshkiragg -q-aeaac-q -seveavslg
H318/3  alpha1a     -scapkspac rtrsppgars -q-qraps-q -wrlcp*...
Rat     alpha1a     -ttqlrakvs slshkirs.g -r-aetac-l -seveavsln
        Consensus   P--------- ---------- A-R-----A- R------SL-

                    561                                   588
human   alpha1a     vphevaegat cqayeladys nlretdi*
H318/3  alpha1a     .......... .......... ........
Rat     alpha1a     vpqdgaeavi cqayepgdys nlretdi*
        Consensus   VP---AE--- CQAYE--DYS NLRETDI*
```

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FIGURE 5A, 29/37
FIGURE 5B, 30/37
FIGURE 5C, 31/37
FIGURE 5D, 32/37

FIGURE 5A

```
                        1                                                  40
Rat       alpha1b       ---------- -----h---- -dd------- ----------
Hamster   alpha1b       ---------- -----q---- -da------- ----------
Human     alpha1b       ---------- -----h---- -na------- ----------
          Consensus     MNPDLDTGHN TSAPA-WGEL K--NFTGPNQ TSSNSTLPQL

                        41                                                 80
Rat       alpha1b       -v-------- ---------- ---------- ----------
Hamster   alpha1b       -v-------- ---------- ---------- ----------
Human     alpha1b       -i-------- ---------- ---------- ----------
          Consensus     D-TRAISVGL VLGAFILFAI VGNILVILSV ACNRHLRTPT

                         81                                                120
Rat       alpha1b       --------i- ---------- ---t------ ----------
Hamster   alpha1b       --------i- ---------- ---t------ ----------
Human     alpha1b       --------m- ---------- ---a------ ----------
          Consensus     NYFIVNLA-A DLLLSFTVLP FSA-LEVLGY WVLGRIFCDI

                        121                                                160
Rat       alpha1b       ---------- ---------- ---------- ----------
Hamster   alpha1b       ---------- ---------- ---------- ----------
Human     alpha1b       ---------- ---------- ---------- ----------
          Consensus     WAAVDVLCCT ASILSLCAIS IDRYIGVRYS LQYPTLVTRR
```

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FIGURE 5B

```
                    161                                                 200
Rat      alpha1b    ---------- ---------- ---------- ----------
Hamster  alpha1b    ---------- ---------- ---------- ----------
Human    alpha1b    ---------- ---------- ---------- ----------
         Consensus  KAILALLSVW VLSTVISIGP LLGWKEPAPN DDKECGVTEE

                    201                                                 240
Rat      alpha1b    --c---c--- ---------- ---------- ----------
Hamster  alpha1b    --y---s--- ---------- ---------- ----------
Human    alpha1b    --y---s--- ---------- ---------- ----------
         Consensus  PF-ALF-SLG SFYIPLAVIL VMYCRVYIVA KRTTKNLEAG

                    241                                                 280
Rat      alpha1b    ---------- ---------- ---------- ----------
Hamster  alpha1b    ---------- ---------- ---------- ----------
Human    alpha1b    ---------- ---------- ---------- ----------
         Consensus  VMKEMSNSKE LTLRIHSKNF HEDTLSSTKA KGHNPRSSIA

                    281                                                 320
Rat      alpha1b    ---------- ---------- ---------- ----------
Hamster  alpha1b    ---------- ---------- ---------- ----------
Human    alpha1b    ---------- ---------- ---------- ----------
         Consensus  VKLFKFSREK KAAKTLGIVV GMFILCWLPF FIALPLGSLF
```

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FIGURE 5C

```
                    321                                                 360
Rat       alpha1b   ---------- ---------- ---------- ----------m
Hamster   alpha1b   ---------- ---------- ---------- ----------m
Human     alpha1b   ---------- ---------- ---------- ----------v
          Consensus STLKPPDAVF KVVFWLGYFN SCLNPIIYPC SSKEFKRAF-

                    361                                                 400
Rat       alpha1b   --------..- gg-------- --a------- ----------
Hamster   alpha1b   --------..- sg-------- --a------- ----------
Human     alpha1b   --------rg- gr-------- --g------- ----------
          Consensus RILGCQC--R --RRRRRRRR LG-CAYTYRP WTRGGSLERS

                    401                                                 440
Rat       alpha1b   ---------- ---m--qk-- ---------- ----tq--v-
Hamster   alpha1b   ---------- ---m--sq-- ---------- ----aq--l-
Human     alpha1b   ---------- ---l--sq-- ---------- ----ap--v-
          Consensus QSRKDSLDDS GSC-SG--RT LPSASPSPGY LGRG--PP-E

                    441                                                 480
Rat       alpha1b   ---f----.p ------..-- -------l-- ---------g
Hamster   alpha1b   ---y----.s ------..-- -------l-- ---------g
Human     alpha1b   ---f----ap ------pa-- -------h-- ---------t
          Consensus LCA-PEWK-- GALLSL--PE PPGRRGR-DS GPLFTFKLL-
```

FIGURE 5D

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```
                    481                                                 520
Rat       alpha1b   d------eat ------dttt -l-------- -----g--h-
Hamster   alpha1b   e------egd ------datt -l-------- -----a--h-
Human     alpha1b   e------dgg ------eaaa -v-------- -----a--q-
          Consensus -PESPGT--- ASNGGC---- D-ANGQPGFK SNMPL-PG-F

                    521
Rat       alpha1b   *
Hamster   alpha1b   *
Human     alpha1b   *
          Consensus *
```

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FIGURE 6A

FIGURE 6A, 33/37
FIGURE 6B, 34/37
FIGURE 6C, 35/37


```
                    1                                                  40
Human    alpha1c    ---------- -----q--a- ---------- ----------
Bovine   alpha1c    ---------- -----h--p- ---------- ----------
         Consensus  MVFLSGNASD SSNCT-PP-P VNISKAILLG VILGGLILFG

                    41                                                 80
Human    alpha1c    ---------- ---------- ---------- ----------
Bovine   alpha1c    ---------- ---------- ---------- ----------
         Consensus  VLGNILVILS VACHRHLHSV THYYIVNLAV ADLLLTSTVL

                    81                                                120
Human    alpha1c    -------v-- ---------- i--------- ----------
Bovine   alpha1c    -------i-- ---------- v--------- ----------
         Consensus  PFSAIFE-LG YWAFGRVFCN -WAAVDVLCC TASIMGLCII

                    121                                               160
Human    alpha1c    ---------- ---------- r--------- ----------
Bovine   alpha1c    ---------- ---------- k--------- ----------
         Consensus  SIDRYIGVSY PLRYPTIVTQ -RGLMALLCV WALSLVISIG
```

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FIGURE 6B

```
                    161                                                 200
Human    alpha1c    ---------- ---------- ---------- ----l--a--
Bovine   alpha1c    ---------- ---------- ---------- ----v--t--
         Consensus  PLFGWRQPAP EDETICQINE EPGYVLFSAL GSFY-PL-II

                    201                                                 240
Human    alpha1c    ---------- ---------- ---------- ----------
Bovine   alpha1c    ---------- ---------- ---------- ----------
         Consensus  LVMYCRVYVV AKRESRGLKS GLKTDKSDSE QVTLRIHRKN

                    241                                                 280
Human    alpha1c    -pa----ma- --t------- ---------- ----------
Bovine   alpha1c    -qv----vt- --n------- ---------- ----------
         Consensus  A--GGSG--S AK-KTHFSVR LLKFSREKKA AKTLGIVVGC

                    281                                                 320
Human    alpha1c    ---------- ---------- -k-------- v---------
Bovine   alpha1c    ---------- ---------- -r-------- a---------
         Consensus  FVLCWLPFFL VMPIGSFFPD F-PSETVFKI -FWLGYLNSC
```

FIGURE 6C

```
                    321                                                 360
Human    alpha1c    ---------- ---------- ------c--- ----a-----
Bovine   alpha1c    ---------- ---------- ------r--- ----t-----
         Consensus  INPIIYPCSS QEFKKAFQNV LRIQCL-RKQ SSKH-LGYTL

                    361                                                 400
Human    alpha1c    -p--qav--- ---m------ -r----r--- --------f-
Bovine   alpha1c    -a--hvl--- ---l------ -a----k--- --------i-
         Consensus  H-PS---EGQ HKD-VRIPVG S-ETFY-ISK TDGVCEWK-F

                    401                                                 440
Human    alpha1c    --m------i t-sk-q-s-- ---------- -----v----
Bovine   alpha1c    --l------m a-ar-p-a-- ---------- -----l----
         Consensus  SS-PRGSAR- -V--D-S-CT TARVRSKSFL QVCCC-GPST

                    441                                 467
Human    alpha1c    --ldk---v- ---v------ ------*
Bovine   alpha1c    --hge---i- ---i------ ------*
         Consensus  PS---NHQ-P TIK-HTISLS ENGEEV*
```

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FIGURE 7A

FIGURE 7A
FIGURE 7B, 36/37
FIGURE 7C, 37/37

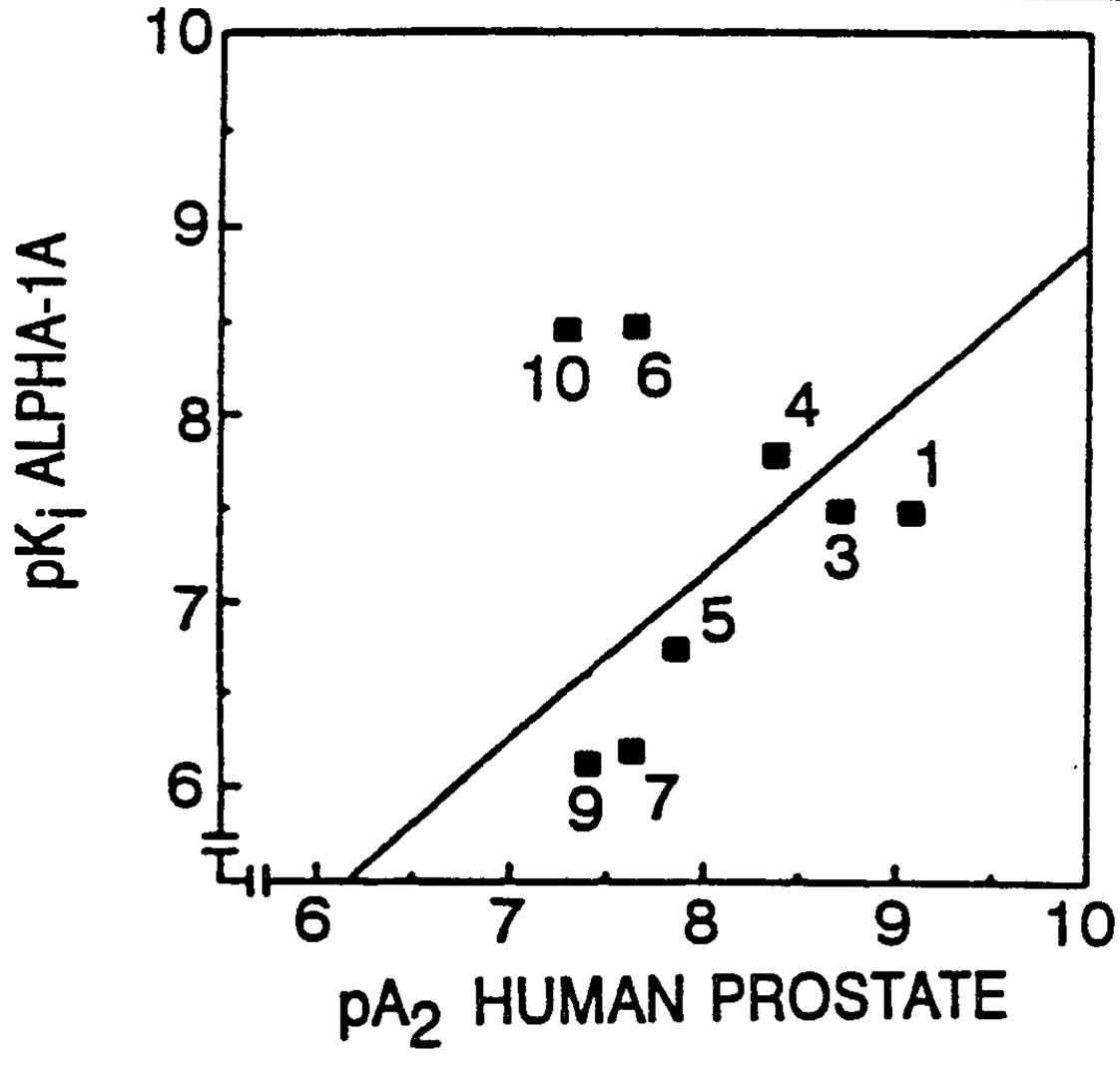
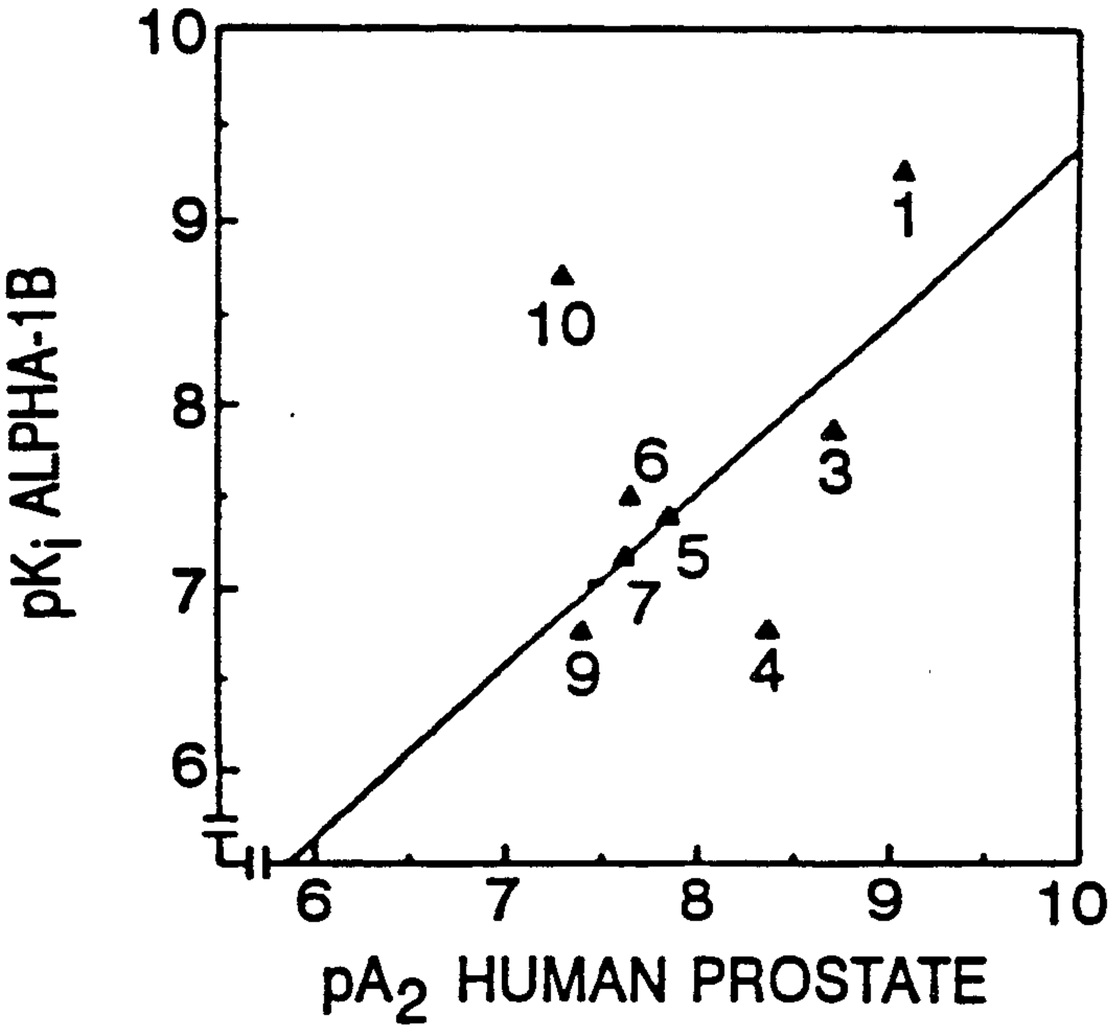


FIGURE 7B



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FIGURE 7C

