

31829-00

RECEPTOR IDENTIFICATION METHOD

5 The superfamily of G protein-linked receptors controls many physiological functions. These receptors mediate transmembrane signaling from external stimuli (vision, taste and smell), endocrine function (pituitary and adrenal), exocrine function (pancreas), heart rate, lipolysis, and carbohydrate metabolism. The molecular cloning of only a fraction of the genes for this family has revealed many structural and genetic similarities including seven membrane spanning domains, conserved amino acids critical for function, and sites for glycosylation and phosphorylation. In many cases, the genes for these receptors lack introns, a rarity among eukaryotic genes. The G protein-linked receptor superfamily can be subclassified into five distinct groups: (i) amine receptors (serotonin, adrenergic, etc.); (ii) small peptide hormone (somatostatin, TRH, etc.); (iii) large peptide hormone (LH-CG, FSH, etc.); (iv) secretin family; and (v) odorant receptors (2).
10 The isolation of as yet uncloned receptors that are associated with important physiological functions or disease states, or which are critical for enhancement of animal performance [e.g., receptors for growth hormone release factor, corticotropin release factor, cholecystokinin, vasoactive intestinal peptide (high affinity subtype)] would be highly beneficial.

25 Current strategies for cloning members of this receptor superfamily are diverse and fraught with

difficulties. These techniques require the isolation of a full-length cDNA or gene to determine whether a particular protein is actually the receptor of interest. For example, expression cloning using a heterologous system (such as Xenopus oocytes) requires the isolation of the full-length cDNA (15). The use of PCR with degenerate oligonucleotides has also been used to clone G protein-linked receptors. However, this approach has been largely applied only to amine receptors, and not to peptide hormone receptors (9). A major drawback to that methodology is the generation of "orphan" receptors where the identification of the receptor, via identification of the hormone that activates it, may remain unknown for years. Finally, protein purification is difficult because these receptors are, in general, very low in abundance. Obtaining peptide sequence data is, therefore, an arduous and time-consuming task. In contrast, the present application describes a system that does not require isolation of a full-length cDNA until a PCR fragment has already been identified as originating from that gene. Additionally, utilization of the highly processive PCR technique, in combination with other steps, circumvents any difficulties due to low abundance.

SUMMARY OF THE INVENTION

The present invention relates to a method for isolation and identification of novel receptor DNA. The method comprises steps of (a) contacting a template nucleic acid, in a polymerase chain reaction, with at least one target receptor-specific oligonucleotide primer, and isolating a nucleic acid fragment resulting from the reaction; (b) providing an antisense oligonucleotide based on a nucleotide sequence from the isolated fragment to a cell capable of expressing a particular receptor, and (c) observing the presence or

absence of the particular receptor's expression or function, in that cell. The absence of receptor expression indicates that the antisense oligo has blocked that receptor's expression, thereby confirming the identity of the isolated nucleic acid fragment upon which the antisense oligo was based as encoding all or a portion of the receptor whose expression has just been measured.

The present method is useful for identifying receptors from virtually any class, but is particularly useful in identification and isolation of G protein-linked receptor. For use in identification of this receptor type, the invention also provides a series of several novel oligonucleotides useful as primers in the first step polymerase chain reaction. The method has also resulted in the identification of a heretofore unknown somatostatin receptor subtype, herein designated as SSSTR3; the invention therefore also provides a nucleic acid sequence encoding that receptor, as well as vectors and host cells capable of expressing the sequence.

The present invention provides significant advantages over known methods of receptor sequence isolation and cloning. It provides an alternative to the process of isolating receptor protein in order to obtain sequence information, a task which is necessarily arduous because of the frequently small amounts of expressed receptor in most cells; the use of PCR in the present method overcomes this problem. The ease of identification of the present method also avoids the classic problem of "orphan receptors", i.e., isolated nucleic acid fragments presumed to encode a receptor protein, but the identity of which remains unknown for lack of a reliable assay and/or a complete sequence. The claimed method provides a means for immediately identifying the ligand specificity of the isolated fragment by quick assay of its activity in wild-type



cells and further avoids the necessity for having a full-length cDNA clone for heterologous expression in a host cell.

BRIEF DESCRIPTION OF THE FIGURES

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Figure 1. Procedure Summary. In this example, four orphan G protein-linked receptor gene fragments are isolated. Vehicle, sense (+; as a negative control) and antisense oligos (-) derived from each clone are tested in a cAMP accumulation assay to determine if one attenuates the response to a ligand known to activate adenylyl cyclase. In this case, an antisense oligo to orphan #3 represses the response, thereby identifying that sequence as a portion of the gene encoding the assayed receptor.

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Figure 2. Description of oligonucleotides used as PCR primers. Lines with boxes represent genes encoding G protein-linked receptors. Each box indicates a transmembrane domain. Arrows correspond to binding sites for the described oligos. Nucleotide sequences use the IUPAC-IUB nomenclature for incompletely specified bases. A. Oligonucleotides directed toward the general class of peptide ligand receptors. B. Oligonucleotides directed toward the secretin receptor-like subclass.

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Figure 3. Nucleotide sequences of orphan receptor DNA fragments. Underlined sequences indicate PCR primers. Both fragments contain oligo #8 at the 5' terminus and oligo #10 at the 3' terminus.

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Figure 4. Hydropathy plots of peptides encoded by novel PCR fragments. The peptide product of each PCR fragment is analyzed by the Kyte-Doolittle hydropathy index. Each sequence begins within the second transmembrane domain and ends in the sixth transmembrane domain. Hydrophobic transmembrane domains 3, 4, and 5 are readily apparent. The plot

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derived from the comparable region of a rat somatostatin (SRIF) receptor is shown for comparison.

5 Figure 5. Antisense oligos block expression of
somatostatin receptors in GH₄C₁ cells: analysis by
radioligand binding. GH₄C₁ cells are treated with
vehicle (CONTROL), an antisense DNA or the correspond-
ing sense strand for 24 hours as described in the text.
The cells are harvested, crude plasma membranes are
prepared and radioligand binding is performed using
10 [¹²⁵I]somatostatin (50 fmol/tube, 250 pMol final).
Non-specific binding is determined in the presence of
1 μM somatostatin. The results displayed are the
average of two separate experiments, performed in
triplicate.

15 Figure 6. Antisense oligonucleotide inhibition of
somatostatin receptor function in AtT20 corticotrophs.
AtT20 corticotrophs are treated with vehicle (control),
a sense oligo (5 μM), or an antisense oligo (5 μM)
directed against the orphan G-protein-linked receptor
PCR11 for 24 hours. The cells are harvested and
20 somatostatin-mediated inhibition of cyclic AMP response
curve performed three times with equivalent results.
The lower panel is the average of three separate
experiments.

25 Figure 7. Nucleotide sequence of mouse SSTR3 DNA
fragment. Underlined sequences indicate PCR primers.
Oligo #8 anchors the 5' terminus and oligo #12 is the
3' terminus.

20 Figure 8. Alignment of somatostatin receptor
peptides. Amino acid sequences of SSTR1 and SSTR2 are
aligned to SSTR3. Lines above the sequences indicate
hydrophobic transmembrane domains. Only sequences from
transmembrane region 2 to transmembrane region 7 are
shown. Small case letters indicate conservative amino
acid substitutions; upper case letters indicate
35 nonconservative changes. To maximize the alignments,
three residues near the C-terminus of transmembrane

domain 4 of SSTR1 and SSTR2 are deleted. Additionally, SSTR1 has 1 fewer residue in tm6 and 3 fewer in tm7.

DETAILED DESCRIPTION OF THE INVENTION

5 The present method can be employed to isolate and
clone nucleotide sequences encoding proteins from any
family of proteins for which (1) a consensus sequence
or sequences can be identified; (2) a cell line ex-
pressing the protein is available; and (3) a functional
10 assay exists. However, the method is particularly
useful in isolation of the nucleotide sequences encod-
ing various types of receptors. Although the specific
examples provided in this application relate to isola-
tion and identification of a nucleic acid fragment
15 encoding a G protein-linked receptor for a small
peptide hormone, those skilled in the art will readily
recognize how the techniques described herein can be
applied to other receptors as well.

 The first step of the method employs the poly-
20 merase chain reaction (hereinafter "PCR") to amplify
the amount of receptor nucleic acid available for
study. The nucleic acid used in the PCR is not re-
stricted to any particular source, but the source cells
will preferably be obtained from tissue which is known
25 to express a particular receptor or receptors of
interest. Tissue sources for various receptors are
well known in the art, as seen, for example in Trends
in Pharmacological Sciences, Receptor Supplement, Jan.
1992 (24). Once an appropriate target cell or tissue
20 type is identified, genomic DNA, cDNA, either from a
library or synthesized from RNA, or RNA itself is
isolated therefrom by standard methods (20), in prepa-
ration for use as a template for PCR.

 The technique of PCR is described in U.S. Patent
35 Nos. 4,683,202; 4,683,195; and 4,965,188; the contents
of these patents are incorporated herein by reference.

In brief, PCR is useful in amplifying, cloning and/or detecting a target sequence in a sample of DNA. Typically, the native duplex DNA strand is denatured, so that the individual strands separate. The separate strands each constitute a template which is contacted with primers, which are usually oligonucleotides designed to be able to anneal with a portion of the target sequence contained on the template. The primers of each strand are then extended by the use of DNA polymerase, thereby providing two new copies of the target sequence. The entire process is repeated numerous times, thus potentially resulting in millions of copies of the targeted sequence, making detection of a gene expressed at very low levels considerably easier.

As applied to the present method, the template nucleic acid is derived from a cell known or expected to carry nucleic acid encoding one or more receptors of interest. The nucleic acid is then contacted with primers (oligos) specifically designed to anneal with sequences characteristic of the receptor or receptors of interest. For example, in the examples provided herein, comparison of the known nucleotide sequences (see generally 19), particularly of somatostatin (25), substance K (23), substance P (23, 6, 26, 4, 21), neuromedin K (22), thyrotropin (8, 9, 17, 19), LH/hCG (12, 14, 16), and other receptors designated mas (27), mrg and rta indicates from regions exhibiting a significant degree of conservation. From this observation, five oligonucleotide primers are designed; with 4- to 64-fold degeneracy plus 5-23% inosines. (See Figure 2A.) These oligos are capable of recognizing virtually any receptor for a peptide ligand in the PCR procedure, and indeed, as shown below, are used to isolate the sequence encoding a novel somatostatin receptor. Other heretofore unisolated receptor genes which may be cloned using these oligos include, but are

not limited to, the receptors, CRF (corticotropin release factor), GNRH (gonadotropin release hormone), follitropin release hormone, growth hormone releasing hormone, octopamine, galanin, adenosine subtypes, angiotensin (II) subtypes, monocyte chemoattractant protein-1, and vasopressin subtypes.

If a different type of receptor is sought, alternate oligos can readily be constructed. For example, to identify genes encoding the secretin type of receptor (which are structurally similar to the peptide hormone receptors, but have quite distinct amino acid sequences), a comparison of the secretin, calcitonin and parathyroid receptor gene sequences provide the information for design of four novel oligos which can be used in recognition of receptors of this subclass (Figure 2B). This group of receptors potentially includes secretin, calcitonin, parathyroid hormone, growth hormone releasing factor (GRF) and glucagon.

For the identification of amine receptors, a number of oligos have already been described (8). Similarly, appropriate sequences for odorant receptors have also been described (2). In each case, these can be used in the same manner as the oligos described above.

The foregoing lists relate to G protein-linked receptors, but oligos for use in this method can also be made for other types of receptors, e.g., non G protein-linked peptide hormone receptors and steroid hormone receptors. The appropriate oligonucleotides can be designed by comparison of several known related genes of that receptor type to determine regions of homology among them, then creating degenerate oligos based on one or more of these consensus sequences.

In the PCR procedure, the number of primers used is not critical and, to some extent, will be governed by the number of useful oligos available for this purpose. Generally, optimal primer number is

determined empirically, dependent on the results obtained with a first pair of primers used. Typically, a balance is struck between the number of oligo pairs, the number of homologous sequences present in each receptor subclass to which oligos may be directed, and the facilities available to analyze the products.

The conditions for PCR reactions are well known in the art. The resulting clones are then isolated and the nucleotide sequence determined for all or part of the clone. At this point, the clone still represents an "orphan", the identity of which remains to be determined. However, it can be determined at this time whether the peptide encoded by the clone has the general hallmarks of a receptor molecule, particularly of the receptor type originally selected for. For example, membrane bound receptors are arranged in a pattern of intracellular (IC), external and transmembrane (TM) domains, though the types of these domains are specific to each group of receptors. The presence of the characteristic domains can be determined from the predicted amino acid sequence by analysis by the Kyte-Doolittle hydropathy index (7; See also Figure 4). Once the protein has been confirmed as likely being a receptor, its subclass identity can be somewhat further refined before proceeding to the next stage of the process. A relatively short (i.e., 8-23 amino acids) third intracellular loop (IC3), for example, is characteristic of G protein-linked peptide ligand receptors; in contrast, the IC3 of amine receptors generally has about 47-155 residues, and contain a conserved Asp residue in their third transmembrane domain. Although this determination is not essential to the success of the method, it will help to screen out clones which are not likely to have been derived from receptor DNA, and to select for those that are likely to be receptor-derived.

In order to more specifically determine the identity of the receptor, the sequence obtained from selected clones is then used to design antisense oligonucleotides. The specific length of the oligo is not critical, but it is preferable to use the largest oligo that can be taken up by cells to provide the greatest sequence specificity. Typically oligos comprise 15 to 21 nucleotides. Generally, at least two complementary oligonucleotides are made, one in the sense orientation and one in the antisense orientation. Preferably the antisense oligos are designed to correspond to regions of mRNA that display the least amount of predicted secondary structure. Each oligo preferably contains 50-70% cytosines plus guanines to maximize hybridization to mRNA. The antisense oligo is used to evaluate whether any receptor function can be blocked in a wild-type cell. If the orphan clone from which the antisense sequence is derived does represent all or part of a receptor gene, then the antisense oligo (but not the sense oligo), when provided to a cell normally capable of expressing that same receptor, should block expression of that receptor.

Since the desired subclass of receptor is determined at the start of the procedure, the type of cell used to evaluate the effect of the antisense oligo will be selected from among cells known to normally express receptors of the type and subclass initially sought. Whole cells or cell membranes from the selected cell type are treated with either an antisense oligo, its complementary sense oligo, or vehicle (as a control). The treated preparations are then observed to determine the presence or absence of receptor function in the presence of a ligand which normally binds to the receptor. In a typical situation, a variety of ligands are tested; the ligands selected for testing are those which are known to bind to receptors within the subclass sought.

The activity of a receptor in the presence of its ligand can be determined in a number of ways. The ligand can be detectably labelled, e.g., with a radioisotope, chemiluminescent molecule, or any other detectable label commonly used in the art. The labelled ligand is then contacted with the treated cells, or preferably cell membranes from treated cells, and the presence or absence of binding of the ligand to the cell or cell membranes determined. The observation of ligand binding in an antisense-treated cell indicates that the antisense oligo had no effect on expression of that ligand's receptor, thereby eliminating that receptor from consideration as being the one encoded by the orphan clone. On the other hand, the absence or reduction of bound ligand to cell membranes from antisense treated cells, combined with the observation of ligand binding in the sense-treated cell membranes, indicates that the original orphan clone represents all or a portion of the gene encoding the receptor for that particular ligand.

Alternately, assuming that the receptors sought are of the type whose action is mediated by a second messenger, then receptor function can be evaluated by measuring accumulation of the second messenger. For example, many peptide hormones mediate their ultimate effects by way of the action of cyclic AMP (cAMP). Thus, the binding of a particular ligand to a receptor can be evaluated by measuring the accumulation of cAMP within antisense-treated intact cells. If there is no stimulation or inhibition (depending on the receptor) of cAMP accumulation in an antisense-treated cell in the presence of a particular ligand, then it can be assumed that the receptor for that ligand was not expressed, and that the orphan sequence shares identity with the receptor for that ligand. Cyclic cAMP accumulation assays are well known in the art (3, 5). However, the practice of the invention is not limited

to identification of receptors for peptide hormones where activity is mediated by cAMP. Other second messenger systems are also known, e.g., ion channels are detected by patch clamp, and inositol phosphates and arachidonic acid can be assayed using commercially available kits (New England Nuclear). In the case in which second messenger accumulation is used as the central indication of receptor blockage, labelled ligand binding can be used as a further confirmation of receptor identity.

If it should be the case that the original clone represents only a fragment of the receptor, further screening is necessary to isolate the entire gene. This can be easily achieved at this stage, however, because the fragment has already been conclusively demonstrated to be receptor-derived, and therefore can be reliably utilized as a probe to screen genomic or cDNA libraries for the entire receptor sequence. Alternately, a method for rapid amplification of cDNA ends (RACE; 11) permits cloning of a complete gene sequence without library screening. Sequencing and subsequent cloning of the entire sequence can be achieved using standard techniques available in the art (20). Utilizing the foregoing method, a novel somatostatin receptor is identified.

It will be understood by those skilled in the art that the present method is applicable to a wide variety of receptors. Among the receptors of particular interest are the receptors for GRF, VIP, galanin, glucagon, β -endorphin, CCKB, GHRH, GNRH, follitropin releasing hormone, CRF, octopamine, adenosine subtypes, angiotensin II subtypes, monocyte chemoattractant protein-1, and vasopressin isoforms. There are also convenient, publicly available host cell lines for use in the antisense assay: for example, Rin cells provide a source of expressed glucagon and galanin receptors; RC-4B cells are a useful cell line for GRF, GHRH, GNRH,

and follitropin releasing factor receptors. NG108 cells are a source of β -endorphin receptor. AtT20 cells can be used in connection with CRF receptor. Neuroblastoma cells are a convenient source for CCKB.

In addition to those receptors and cell lines noted above, variations of the claimed method will also be apparent to those skilled in the art.

The invention is further illustrated in the following non-limiting examples.

EXAMPLES

1. General Methods

Unless otherwise specified, the following methods are utilized in the procedures described below:

Polymerase Chain Reaction. Oligonucleotides are synthesized on an Applied Biosystems Model DNA synthesizer. PCR reactions are done with the Perkin-Elmer Cetus GeneAmp® PCR kit if using DNA as the template or with the same manufacturer's GeneAmp® rTth RNA PCR kit if using RNA as the template. Reactions are performed, but not limited to, the manufacturer's recommendations with a 39°C annealing temperature for degenerate oligonucleotides or 60°C for specific primers. Each reaction contains 1 μ g DNA or total cellular RNA plus 1 μ g of each primer (ca. 1 μ M final concentration).

Sequence Analysis. PCR products are separated in a 1.2% agarose slab gel containing 1 μ g/ml ethidium bromide. DNA fragments of the expected length are cut out and purified with GeneClean® (BIO101). The termini are polished with T4 DNA polymerase in the presence of 0.25 mM dNTPs and ligated into pGEM3Z (Promega Corp.) cleaved with SmaI. Selected clones are sequenced using the Applied Biosystems dye primer DNA sequencing kit and the Applied Biosystems Model 373A automated sequencer. Protocols are as recommended by Applied

Biosystems. Nucleotide sequences are analyzed with the MacVector® software package.

2. Isolation of Receptor Fragments

To target peptide receptors, consensus sequences are identified from nine rat genes. These sequences are then utilized to design probes for the isolation of new genes of the peptide receptor class. The receptors used are those for somatostatin, substance K, substance P, neuromedin K, thyrotropin, LH/CG and others designated mas, mrg, and rta. Four regions of nucleotide sequence are found to exhibit a degree of conservation significant enough to design degenerate oligonucleotide primers for PCR. Five oligos are designed with 4- to 64-fold direct degeneracy plus 5% to 23% inosines (Figure 2A). Additional probes are developed for the class of G protein-linked receptors characterized by the rat secretin receptor. This class currently has only four known members (recognizing secretin, calcitonin, parathyroid hormone and vasoactive intestinal peptide). The sequences for these genes are aligned and four oligonucleotides are subsequently designed to potentially recognize this subtype (Figure 2B). Neither set of oligos would be predicted to amplify amine receptor sequences.

The five oligos described in Figure 2A are designed to anneal sequences in G protein-linked peptide receptor genes. Oligos are synthesized on an Applied Biosystems Model DNA synthesizer. The oligos are used as six different primer pairs for PCR. PCR reactions are conducted as described above. Using rat genomic DNA as the template, all six reactions produce DNA fragments of the expected size (ranging from ca. 420 bp for a tm3 to tm6 fragment to ca. 700 bp for a tm2 to tm7 fragment), DNA fragments are cloned and two samples, designated PCR9 and PCR11, are found to contain sequences characteristic of G protein-linked receptors

but no exact matches are found in GenBank (Table 1). Nucleotide sequences of these two fragments are shown in Figure 3. Each fragment contains a single open reading frame. The comparison with other receptor sequences shows a considerable similarity between a rat somatostatin receptor and PCR 11, suggesting that PCR11 is a novel somatostatin receptor subtype. Both fragments exhibit not only primary sequence similarity to other receptors but also the hydropathy pattern characteristic of G protein-linked receptors (Figure 4). Interestingly, the predicted third intracellular loop (IC3) of each new sequence is comparatively short, a hallmark of peptide receptors which have IC3s of 8 to 23 amino acids long (PCR #9 has an IC3 of 13 residues and PCR #11 has one of 23 amino acids). In contrast, the IC3s of amine receptors range from 47 to 155 residues in length. These observations suggest that the novel G protein-linked receptors represented by the PCR products are of the peptide ligand subclass.

TABLE 1
Relatedness of Novel PCR Fragments
to G Protein-Linked Receptors^a

	Receptor Class	Gene Designation ^b	PCR #9 ^c	PCR #11 ^c
5	Peptide Ligand	srif	37	57
		neuK	24	30
		subK	22	25
		subP	20	26
10		mas	23	16
		mrg	15	18
		thy	19	18
		lhcg	16	13
15	Amine Ligand	α 1b	21	27
		β 2	24	20
		d2	10	22

Notes:

- 20 ^a Each PCR fragment (designated #9 or #11) is translated and compared to G protein-linked receptors available in GenBank.
- 25 ^b All sequences are from rat. Designations are: srif, somatostatin receptor; neuK, neuromedin K receptor; subK or P, substance K or P receptor; mas, mas oncogene; mrg, mas related gene; thy, thyrotropin receptor; lhcg, luteinizing hormone and chorionic gonadotropin receptor; α 1b, α _{1b}-adrenergic receptor; β 2, β ₂-adrenergic receptor; d2, D₂ dopamine receptor.
- 20 ^c Numbers represent the percent identical or conserved amino acids over the length of the translated PCR fragment. Any percentage greater than 15 is considered highly significant.

35 3. Blockade of Somatostatin Receptor and Expression
Expression Function with Antisense Oligos

A. Ligand Binding Assay

GH₄C₁ (a pituitary cell line) cells are grown to 50% confluence in 10% fetal bovine serum, Dulbecco's modified Eagles medium in a humidified chamber (37°C, 95:5, air CO₂). The GH₄C₁ cells are then incubated with vehicle (H₂O) (control), with an antisense oligo (18 mer, GGGTCGCCTCCATTTTCGG, 5 μM), or the complementary sense strand (18 mer, GCCAAATGGAGGCGACCC, 5 μM) for 4 hours at 37°C (95:5, air CO₂) in Dulbecco's modified Eagles medium without serum. At the end of the 4-hour time period, heat-inactivated fetal bovine serum is added to 10% (final concentration). The cells are grown for an additional 20 hours. The cells are harvested, crude membranes are prepared as described by Eppler et al. (28). Radioligand binding is performed with [¹²⁵I]tyr-somatostatin, S-14 (50 fmol/tube, 250 pMol final), using standard binding assay methods as follows: binding assays are done in a binding buffer containing 50 mM HEPES (pH 7.4), 0.5% BSA and 5 mM MgCl₂. The standard filtration assay for [¹²⁵I]SRIF analog binding to GH₄C₁ membranes is carried out as follows: 1. Radioligand is diluted in binding buffer + PMSF/Baci to the desired cpm per vol. of 10 μl and then 180 μl aliquots are added to the table. For non-specific binding samples, 10 μl of 20 μM cold S14 is also added per well of membrane diluted to the desired concentration (10-30 ug membrane protein/well) in binding buffer + PMSF/Baci. Binding is stopped by rapid filtration of samples through Whatman GF/C filters presoaked with 0.3% polyethyleneimine. Binding is allowed to proceed at 30°C for 1-2 hours. Binding is stopped by rapid filtration of samples through Whatman GF/C filters presoaked with 0.3% polyethyleneimine. Each sample is washed three times with ice-cold binding buffer (5 ml). Finally the individual wells are placed in 12 x 75 mm tubes and counted in an LKB Gammamaster counter (78% efficiency).

The results, displayed in Figure 5, are the average of two experiments, each performed in triplicate. It can be seen that membrane from cells treated with vehicle and the sense oligo display equivalent levels of binding [125 I]tyr-somatostatin (408 v 350 fmol/mg). In membranes challenged with the antisense oligo for 24 hours, a 60% decline in [125 I]tyr-somatostatin binding is observed (166 mol/mg). Thus blockade of receptor expression with antisense, but not the sense oligos shows a specific means by which loss of function can be used to characterize and identify orphan receptors.

B. cAMP Assay

AtT20 cells are grown to 50% confluence in 10% fetal bovine serum, Dulbecco's modified Eagles medium in a humidified chamber (37°C, 90:10, air:CO₂). The AtT20 cells are then incubated with vehicle (control), with an antisense oligo directed against the orphan G protein-linked receptor PCR11 (20 mer, CGAGCGCCTCCGCCTTGAGG, 5 µM), or the sense strand (20 mer, ACGCAGAACGCCGTCTCCTA, 5 µM) for 4 hours at 37°C (90:10, air:CO₂) in Dulbecco's modified Eagles medium without serum. The mRNA encoding this orphan receptor is detected in AtT-20 mouse pituitary cells by PCR Northern analysis. At the end of the four-hour time period, heat-inactivated fetal bovine serum is added to 10% (final concentration). The cells are harvested and bovine serum is added to 10% (final concentration). The cells are harvested and resuspended in Kreb's Ringer phosphate buffer containing 2 mM CaCl₂, 100 µM isobutyl-methylxanthine (to inhibit cyclic AMP-dependent phosphodiesterase activity). Somatostatin-mediated inhibition of forskolin-stimulated cyclic AMP accumulation is performed in intact cells. Cyclic AMP is measured using a kit purchased from Amersham. Cells are washed two

times with Kreb's Ringer Phosphate (KRP) buffer containing 1 mM CaCl_2 . The cells (50-100,000/ tube in triplicate) are incubated for 15 minutes with activators and/or inhibitors of adenylyl cyclase in KRP buffer (final vol = 100 μl) containing 100 μM isobutylmethylxanthine. The reaction is terminated by addition of 10 μl of 0.1 N HCl. The samples are boiled for 5 minutes and then neutralized with 0.1 N NaOH containing 100 mM Tris, pH 7.5. To each tube [^3H] cyclic AMP is added (~15,000 cpm) and 10 μl of adrenal binding protein. The samples are incubated on ice for 90 minutes with standards, activated charcoal (100 μl) is added, and centrifuged (4°C, 2000 x g for 5 minutes). The samples are compared against standards of known concentrations and quantified by liquid scintillation spectrometry. Results are depicted in Figure 6. The figure shows that the efficacy of somatostatin mediated inhibition of cAMP accumulation is attenuated. Also, the ED50 for the somatostatin mediated response is shifted to the right. In control and sense treated cells, the ED50s for somatostatin receptor are calculated to be 1 and 1.2 nM, respectively. In the cells treated with antisense oligos, the ED50 response to somatostatin is 9 nM, a 9-fold shift in potency. These data indicate that both the efficacy and potency of somatostatin are altered by treatment of cells with antisense oligos. Thus, the PCR11 fragment appears to identify a novel somatostatin receptor subtype. The fragment is compared with known receptor subtypes SSTR1 and SSTR2 (25). Over the region currently available (representing greater than 50% of the coding sequence), the SSTR3 receptor is 57% identical to SSTR2, and is 74% homologous if conservative amino acid substitutions are considered (Figure 8); SSTR3 is only slightly less homologous to SSTR1: 49% identical and 70% identical or conserved residues.

In the course of further experiments, a mouse homologue is also isolated. The nucleotide sequence of this fragment is given in Figure 7.

DEPOSIT OF BIOLOGICAL MATERIALS

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The following materials have been deposited on June 4, 1992, under the terms of the Budapest Treaty, at the American Type Culture Collection, and given the indicated accession numbers.

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<u>Description</u>	<u>Accession No.</u>
E. coli K-12, OZ9 (PCR9)	69005
E. coli K-12, OZ11 (PCR11)	69006

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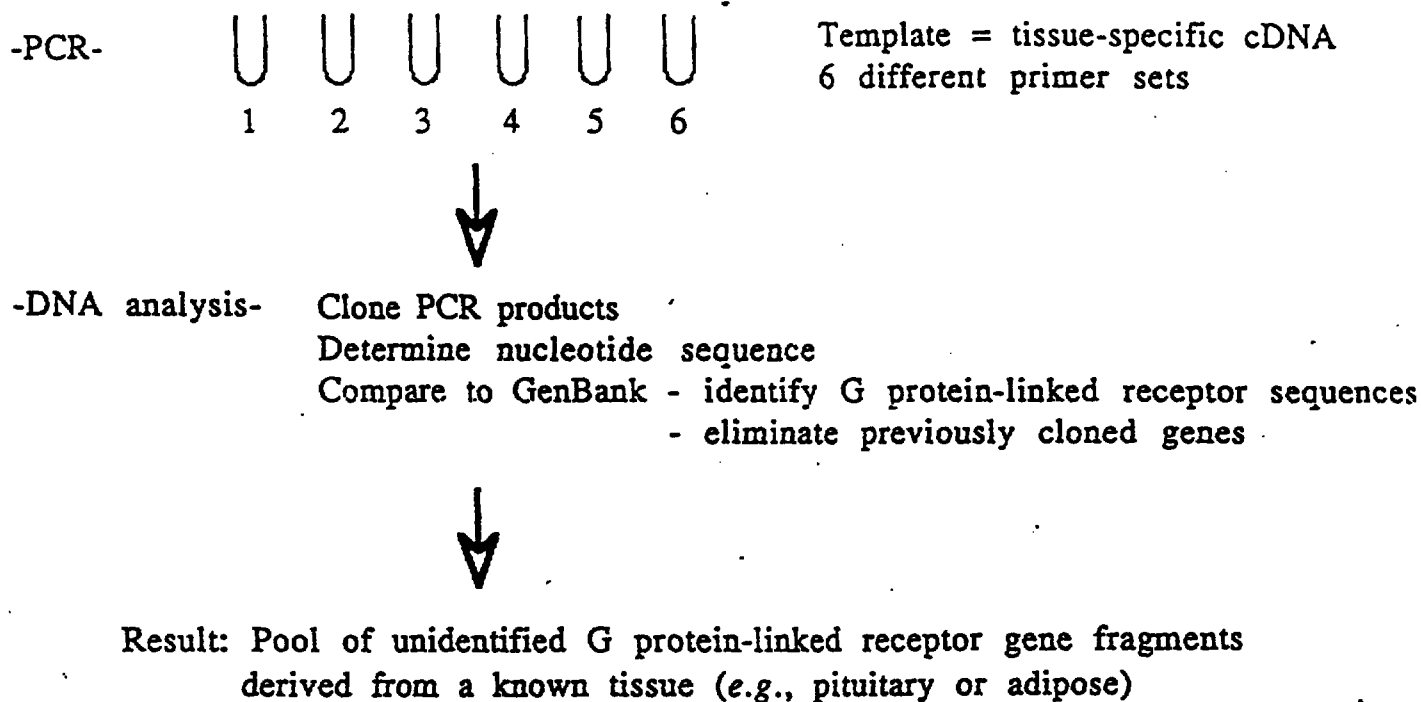
What we claim is:

1. A method for isolation and identification of nucleic acid fragments encoding a receptor comprising: (a) contacting a template nucleic acid, in a polymerase chain reaction, with at least one target receptor-specific oligonucleotide primer, and isolating a nucleic acid fragment resulting from the reaction; (b) providing an antisense oligonucleotide based on a nucleotide sequence from the isolated fragment to a cell capable of expressing a particular receptor; and (c) observing the presence or absence of the particular receptor's expression in that cell.
2. An oligonucleotide selected from the group consisting of the oligonucleotides depicted in Figure 2A.
3. An oligonucleotide selected from the group consisting of the oligonucleotides depicted in Figure 2B.
4. An antisense oligonucleotide having the sequence GGGTCGCCTCCATTTCGG.
5. An antisense oligonucleotide having the sequence CGAGCGCCTCCGCCTTGAGG.
6. A nucleic acid fragment encoding a somatostatin receptor, comprising the amino acid sequence of SSTR3 depicted in Figure 3.



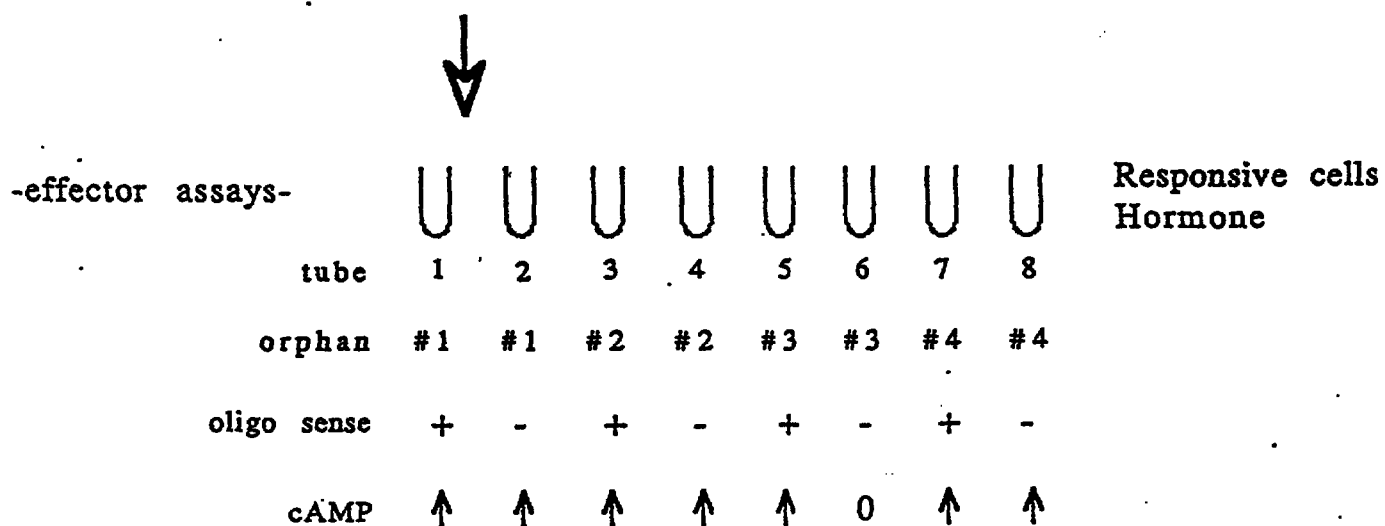
7. A nucleic acid fragment encoding a somatostatin receptor, the fragment comprising the sequence depicted in Figure 7.
8. A nucleic acid fragment encoding a somatostatin receptor, comprising the amino acid sequence of SSTR3 depicted in Figure 8.
9. A nucleic acid fragment comprising the sequence of PCR9, depicted in Figure 3.

Stage 1. PCR & preliminary characterization of DNA fragments.



Stage 2. Antisense oligonucleotide synthesis and effector assays.

-synthesize oligos- Synthesize sense and antisense oligos derived from each orphan
 PCR fragment

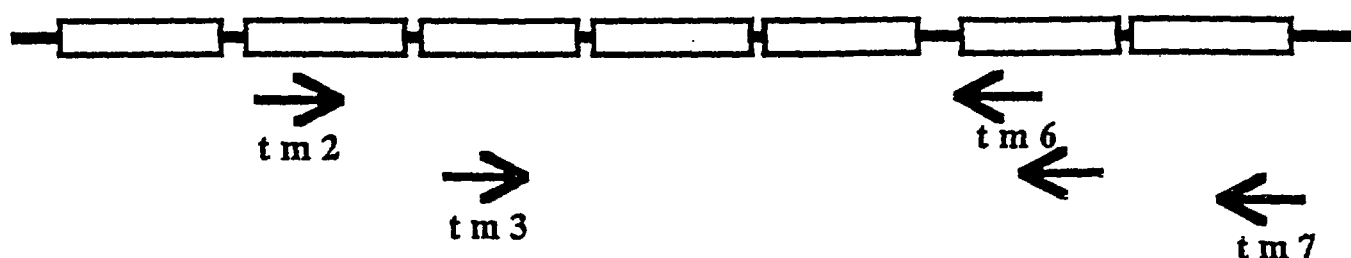


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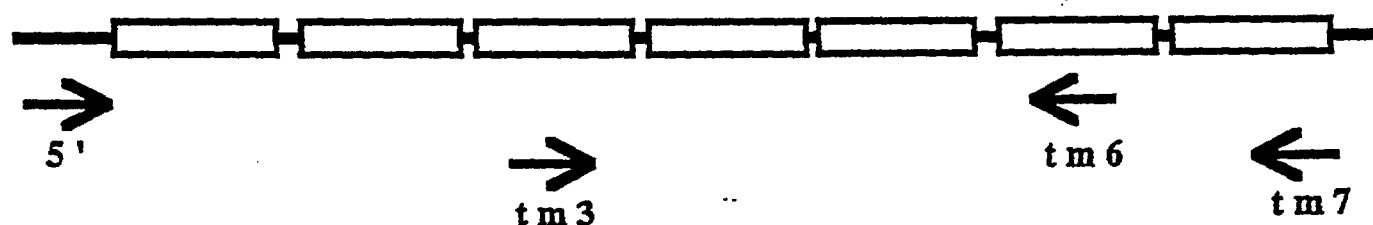
FIGURE 1

A.



Designation	Location	Length	Degeneracy	Nucleotide Sequence
#8	tm2	26	16X; 23% I	TWCITISTIMACCTGGYCIIGCTGA
#9	tm3	25	4X; 12% I	TGTTYICCAGCATCTACTIIMTGAC
#10	tm6	26	64X; 19% I	ATGRTGATSITKGTNGTGIIKKRIIT
#11	tm6	20	4X; 5% I	TTYGYCATCTGCTGGITGOC
#12	tm7	17	8X; 6% I	AAYAGCIGYGCCAASCC

B.



Designation	Location	Length	Degeneracy	Nucleotide Sequence
#13	5'	21	8X; 24% I	TGGGAIRICATKIITGYTGG
#14	tm3	19	4X; 16% I	GAIGGISTCTAICTTCAYA
#15	tm6	19	16X; 21% I	ASTGIAIYICCLARIAGSGG
#16	tm7	22	2X; 23% I	CYTCICCIITGIGAAICAGTA

FIGURE 2

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PCR9

<u>TTCGTGGTGA</u>	<u>ACCTGGTCGG</u>	<u>GGCTGACTTT</u>	CTCCTGATCA	TTTGCTTGCC	GTTCTTGACG	60
GACAACTATG	TCCAGAACTG	GGACTGGAGG	TTCGGGAGCA	TCCCCTGCCG	CGTGATGCTC	120
TTCATGTTGG	CCATGAACCG	ACAGGGCAGC	ATCATCTTCC	TCACGGTGGT	GGCTGTGGAC	180
AGGTACTTCA	GGGTGGTCCA	CCCGCACCAC	TTCCTGAACA	AGATCTCCAA	CCGGACGGCG	240
GCCATCATCT	CTTGCTTCCT	GTGGGGCATC	ACCATCGGCC	TGACAGTCCA	CCTCCTCTAC	300
ACGGACATGA	TGACCCGAAA	CGGCGATGCA	AACCTGTGCA	GCAGTTTTAG	CATCTGCTAC	360
ACTTTCAGGT	GGCAGATGC	AATGTTCTC	TTGGAATTCT	TCCTGCCCT	GGGCATCATC	420
CTGTTCTGCT	CTGGCAGGAT	CATTTGGAGC	CTAAGGCAGA	GACAGATGGA	CAGGCACGTC	480
AAGATCAAGA	GGGCCATCAA	CTTCATCATG	GTGGTTGCCA	TTGTGTTTGC	<u>CATCTGCTGG</u>	540

CTGCC

PCR11

<u>TTCGTGCTGC</u>	<u>ACCTGGTCGG</u>	<u>GGCTGACGTA</u>	TTATTTATGT	TGGGACTTCC	TTTCCTGGCC	60
ACGCAGAACG	CCGTCTCCTA	CTGGCCCTTC	GGCTCCTTCT	TGTGCCGCCT	GGTCATGACA	120
CTGGATGGCA	TCAACCAGTT	CACCAGTATC	TTCTGCCTGA	TGGTCATGAG	TGTTGACCGC	180
TACCTGGCCG	TGGTCCACCC	TCTCCGCTCA	GCCCGGTGGC	GTCGCCACG	GGTAGCCAAG	240
ATGGCCAGCG	CGGCCGTCTG	GGTCTTTTCG	CTGCTCATGT	CTCTGCCGCT	CTTGGTCTTC	300
GCGGATGTCC	AGGAGGGCTG	GGGCACCTGC	AACCTGAGCT	GGCCAGAGCC	TGTGGGGCTG	360
TGGGGTGCAG	CCTTCATCAC	CTACACGTCT	GTGTTGGGCT	TCTTTGGGCC	CCTGCTGGTC	420
ATCTGCTTGT	GCTACCTGCT	CATTGTGGTC	AAGGTGAAGG	CTGCAGGCAT	GCGCGTAGGC	480
TCCTCAAGGC	GGAGACGCTC	GGAGCGCAAG	GTGACTCGCA	TGGTGGTGGT	CGTGGTGCTG	540

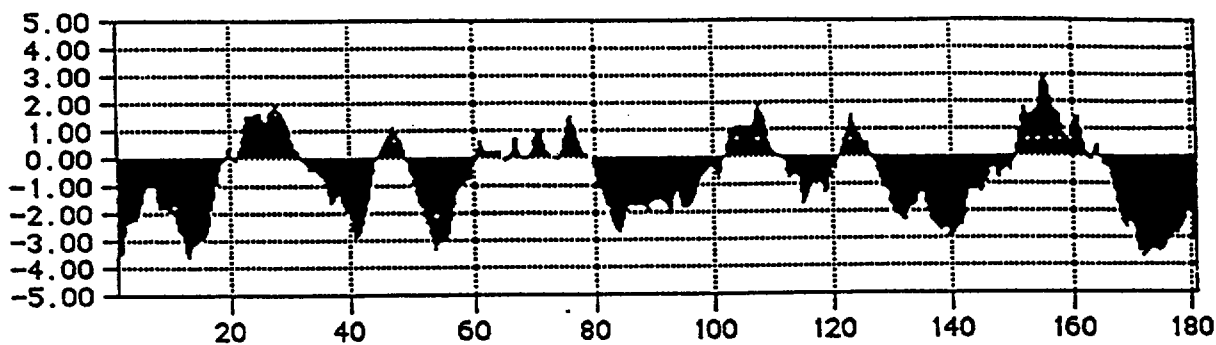
GTGTTGCGCA TCTGCTGGCT GCC

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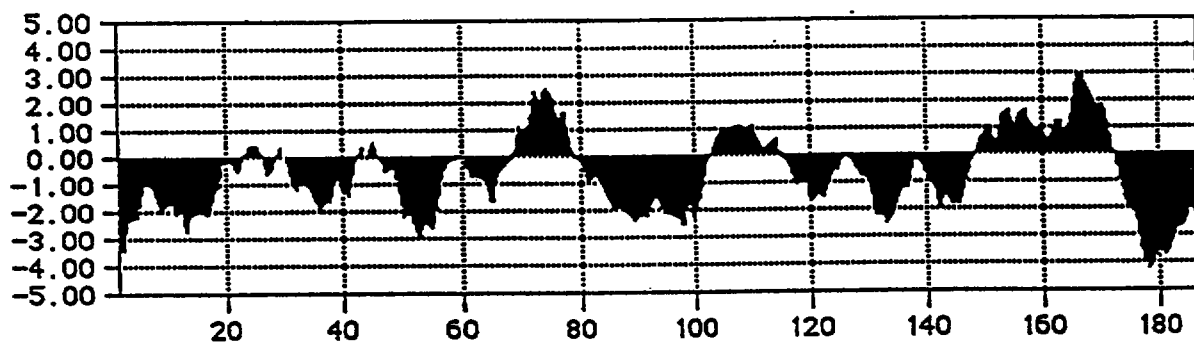


FIGURE 3

PCR9



PCR11



SRIF

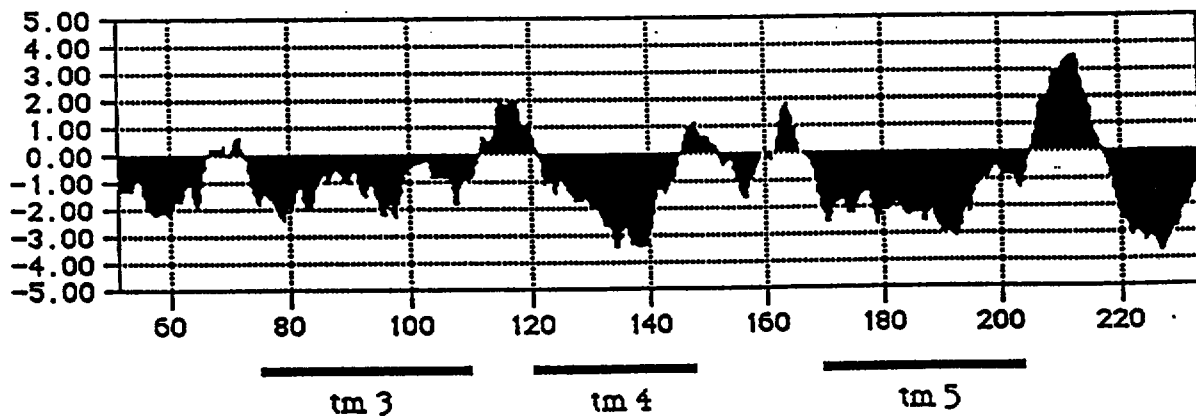


FIGURE 4

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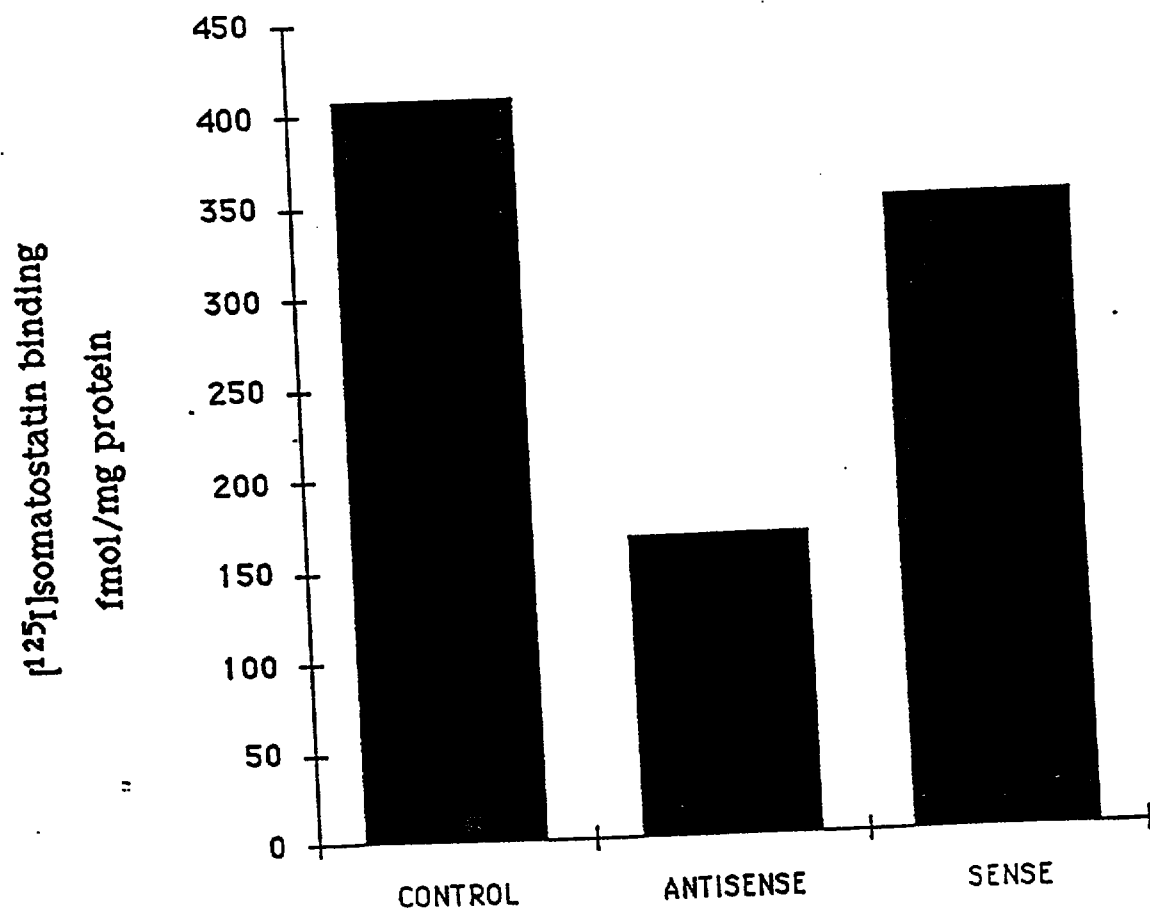
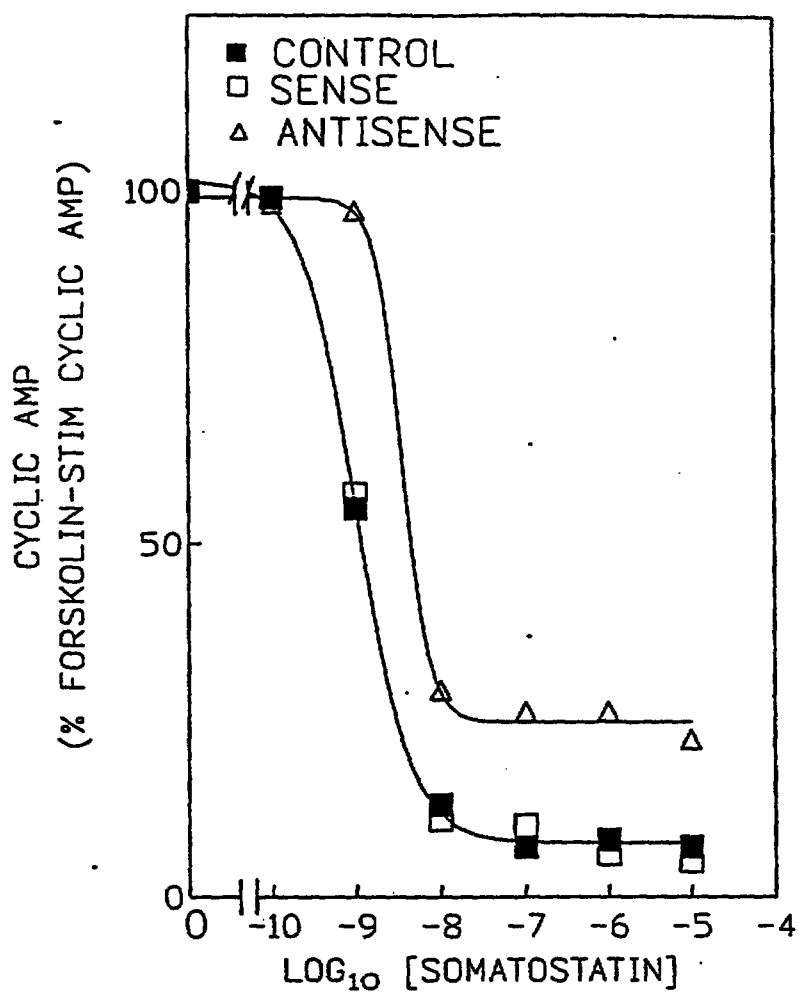


FIGURE 5

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TREATMENT	ED ₅₀ (nM)
CONTROL	0.9
SENSE	1.1
ANTISENSE	7.2

FIGURE 6

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TTCGTGCTGA ACCTGGCCGG GGCTGACGTG TTGTTTATGT TGGGGCTTCC TTCCTGGCA 60
 ACGCAGAATG CTGTCTCCTA CTGGCCCTTT GGCTCCTTCT TGTGCCGCCT GGTCATGACG 120
 CTGGACGGCA TCAACCAGTT CACCAGTATC TTCTGCCTGA TGGTCATGAG TGTCGACCGC 180
 TACCTGGCCG TGGTCCACCC TCTCCGCTCA GCCCGGTGGC GTCGCCCACG GGTAGCCAAG 240
 CTGGCTAGTG CTGCCGTCTG GGTCTTCTCG CTGCTCATGT CTCTGCCGCT CTTGGTCTTT 300
 GCGGATGTCC AGGAGGGCTG GGGCAACTGC AACCTGAGCT GGCCAGAGCC TGTGGGAATG 360
 GGGGTGCAG CCTTCATCAC TTACACGTCT GTGCTGGGCT TCTTTGGGCC CCTGCTGGTC 420
 ATCTGCATGT GCTATTTGCT CATCGTAGTG AAGGTGAAGG CTGCAGGTAT GCGTGTGGGC 480
 TCCTCACGGC GGAGGCGCTC AGAACCCAAG GTGACTCGCA TGGTGGTGGT AGTGGTGCGG 540
 CTGTTTCGTGG GCTGCTGGCT GCCTTTCTTC ATCGTCAACA TCGTCAACCT GGCCTTCACG 600
 CTACCCGAGG AGCCACCTC TGCCGGCCTC TACTTCTTTG TGGTGGTCCT GTCTTATGCC 660
AATAGCCGCG CCAAGCCC

FIGURE 7

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2

3

SSTR3 VLFMLGLPFLATQNAVSYWPFSGFLCRLVMTLDGINQFTS
 2 E-----M-V-lVH---Kai--v---v-----
 1 E-l--Sv---V-STLlRH---Al-----lSv-Av-M---

SSTR3 IFCLMVMSVDRYLAVVHPLRSARWRRPRVAKLASAAVWVF
 2 ----T---i-----ik--k-----T--mINV---CV
 1 -y--T-l-----v-----ikA--Y---T---vVNLG---l

4

SSTR3 SLLMSLPLLVFADVQEGWGNCNLSWPEPVGMWGAAFITYT
 2 ---vI--imiy-GlRNQ--S-TiN--GES-A-YTG--I-A
 1 ---vI--iv---ANSd-TVA--mLM---AQR-LVG-vL--

5

SSTR3 SVLGFFGPLLVICMCYLLIVVKVKAAGMRVGSSRRRRSEP
 2 Fi---lV--Ti--l---f-ii---SS-i-----k-kk--K
 1 Flm--lL-vGA--l--v--iA-mrMVAIka-WQQ-k---R

6

SSTR3 KVTRMVVVVRLFVGCWLPFFIVNIVNLAFTLPEEPTSAG
 2 -----Si--Av-iF-----y-F-vSSvSVAiSPT-ALK-
 1 -i-L--mm--Mv--I--m--yv-Ql--v -AEQdda-VSQ

7

SSTR3 LYFFVVVLSYA
 2 mfd---i-T--
 1 - S-i-G--

FIGURE 8

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☐ Claim(s) :	paged	to paged
☒ Drawing(s) :	paged <u>1/8</u>	to paged <u>5/8</u>
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DATE OF RECEIPT <u>13/8/96</u>		

- 24 -

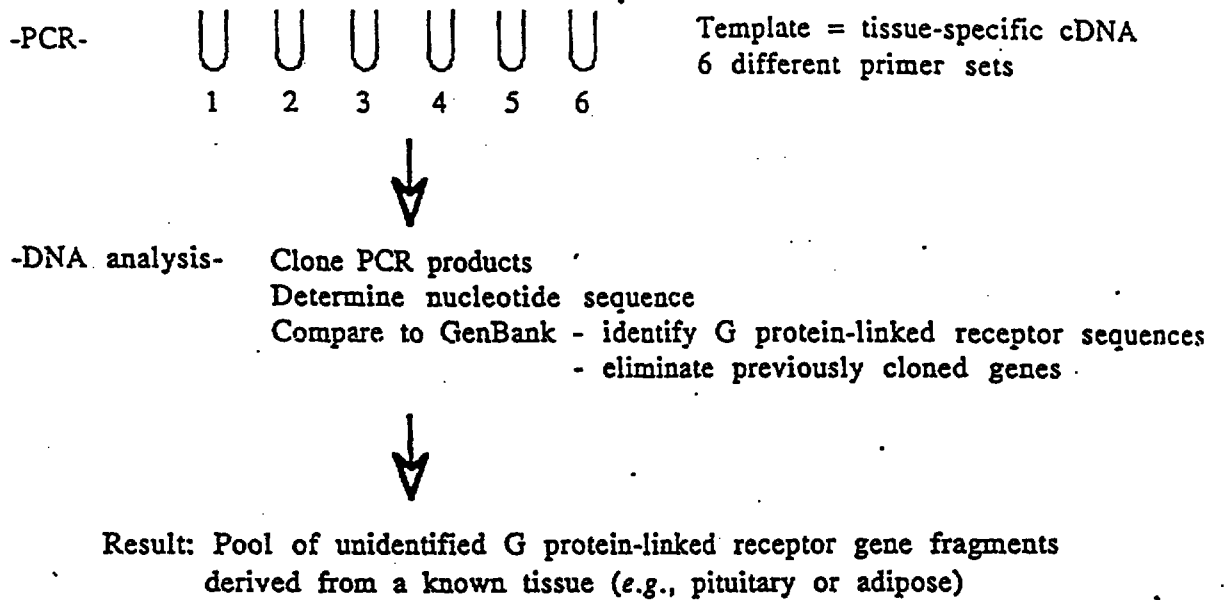
RECEPTOR IDENTIFICATION METHODABSTRACT

The present invention relates to a method for isolating and cloning receptor DNA sequences. The invention also provides novel DNA sequences encoding a novel somatostatin receptor subtype.

Fig. 1

REPLACEMENT SHEET
13 AUG 1996

Stage 1. PCR & preliminary characterization of DNA fragments.



Stage 2. Antisense oligonucleotide synthesis and effector assays.

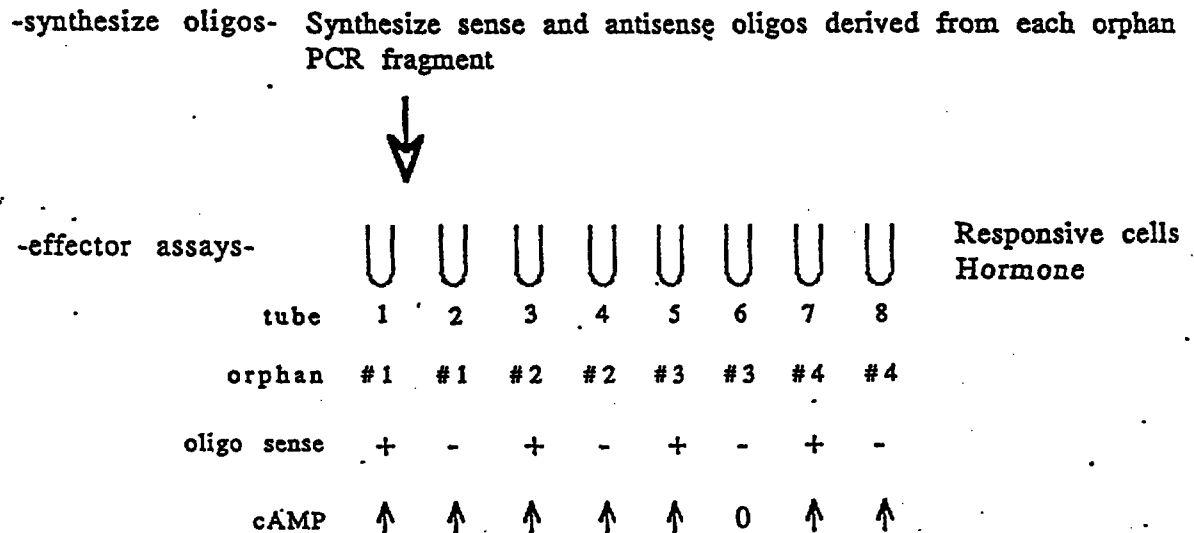
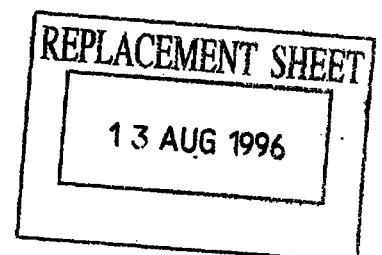


FIGURE 1



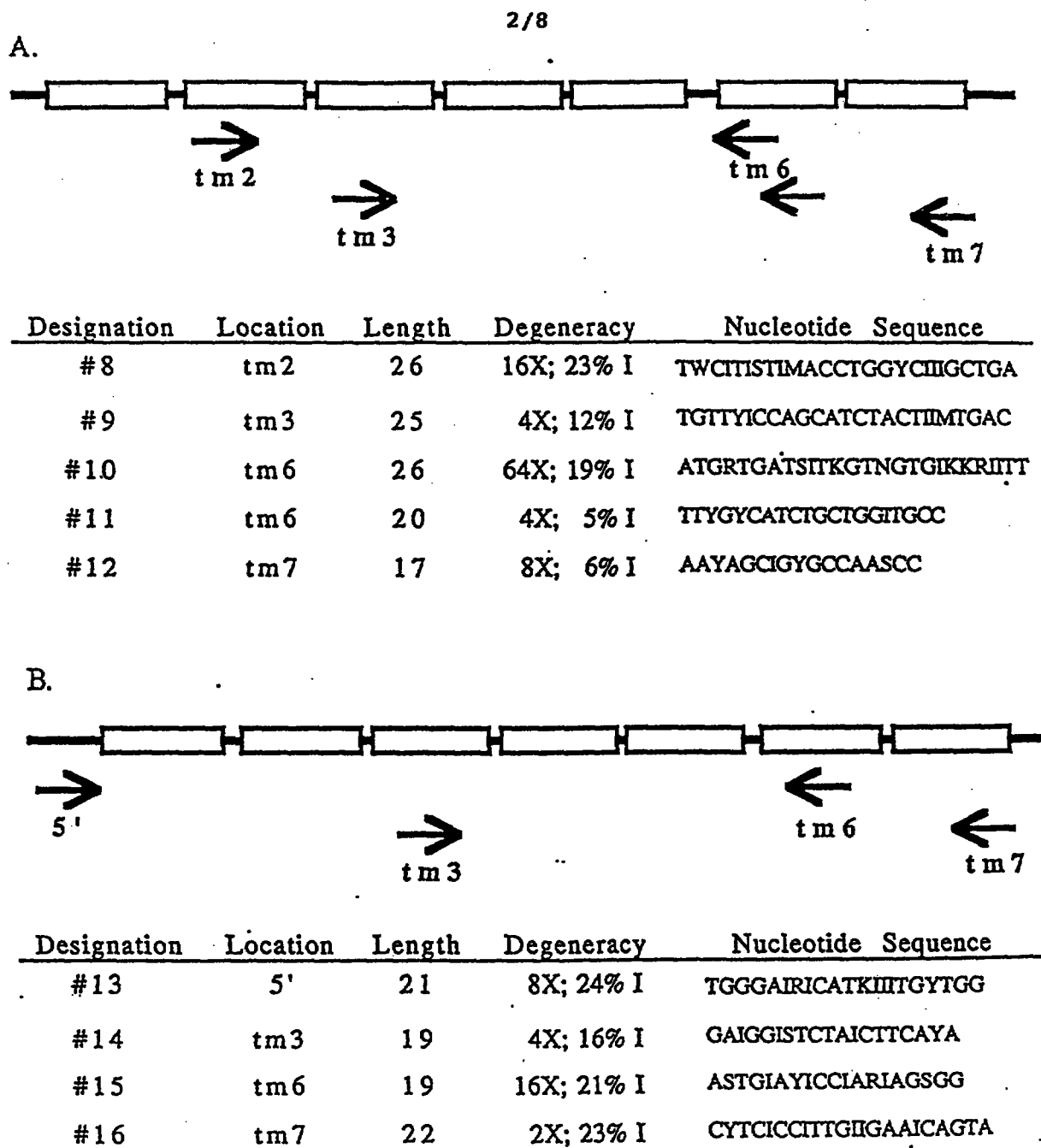
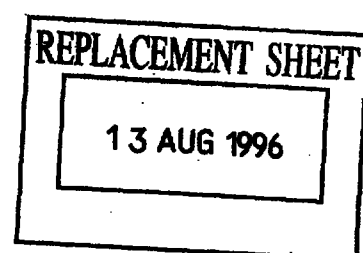


FIGURE 2



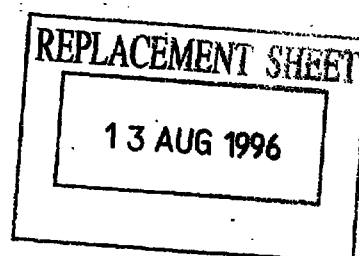
PCR9

TTCGTGGTGA ACCTGGTCGG GGCTGACTTT CTCCTGATCA TTTGCTTGCC GTTCTTGACG 60
 GACAACTATG TCCAGAACTG GGA CTGGAGG TTCGGGAGCA TCCCCTGCCG CGTGATGCTC 120
 TTCATGTTGG CCATGAACCG ACAGGGCAGC ATCATCTTCC TCACGGTGGT GGCTGTGGAC 180
 AGGTACTTCA GGGTGGTCCA CCCGCACCAC TTCCTGAACA AGATCTCCAA CCGGACGGCG 240
 GCCATCATCT CTTGCTTCCT GTGGGGCATC ACCATCGGCC TGACAGTCCA CCTCCTCTAC 300
 ACGGACATGA TGACCCGAAA CGGCGATGCA AACCTGTGCA GCAGTTT TAG CATCTGCTAC 360
 ACTTTCAGGT GGCACGATGC AATGTTCTC TTGGAATTCT TCCTGCCCT GGGCATCATC 420
 CTGTTCTGCT CTGGCAGGAT CATTGAGC CTAAGGCAGA GACAGATGGA CAGGCACGTC 480
 AAGATCAAGA GGGCCATCAA CTTCATCATG GTGGTTGCCA TTGTGTTGC CATCTGCTGG 540
CTGCC

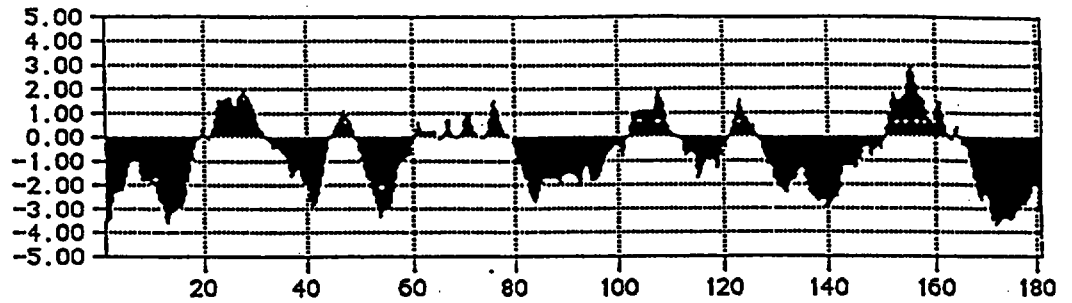
PCR11

TTCGTGCTGC ACCTGGTCGG GGCTGACGTA TTATTTATGT TGGGACTTCC TTTCCTGGCC 60
 ACGCAGAACG CCGTCTCCTA CTGGCCCTTC GGCTCCTTCT TGTGCCGCTT GGTCATGACA 120
 CTGGATGGCA TCAACCAGTT CACCAGTATC TTCTGCCTGA TGGTCATGAG TGTGACCGC 180
 TACCTGGCCG TGGTCCACCC TCTCCGCTCA GCCCGGTGGC GTCGCCACG GGTAGCCAAG 240
 ATGGCCAGCG CGGCCGTCTG GGTCTTTTCG CTGCTCATGT CTCTGCCGCT CTTGGTCTTC 300
 GCGGATGTCC AGGAGGGCTG GGGCACCTGC AACCTGAGCT GGCCAGAGCC TGTGGGGCTG 360
 TGGGGTGCGAG CCTTCATCAC CTACACGTCT GTGTTGGGCT TCTTTGGGCC CCTGCTGGTC 420
 ATCTGCTTGT GCTACCTGCT CATTGTGGTC AAGGTGAAGG CTGCAGGCAT GCGCGTAGGC 480
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GTGTTGCCA TCTGCTGGCT GCC

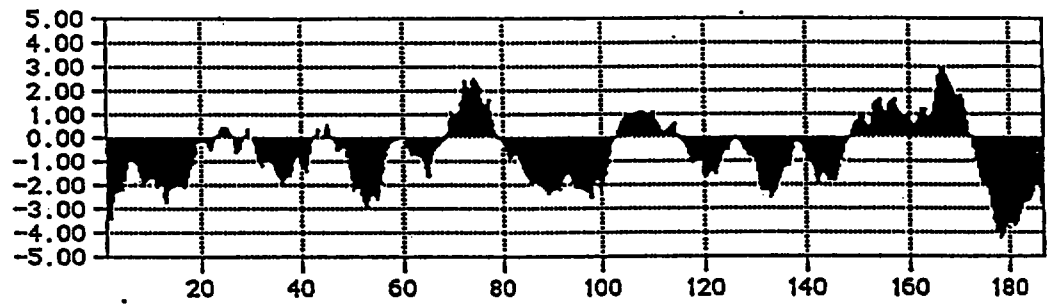
FIGURE 3



PCR9



PCR11



SRIF

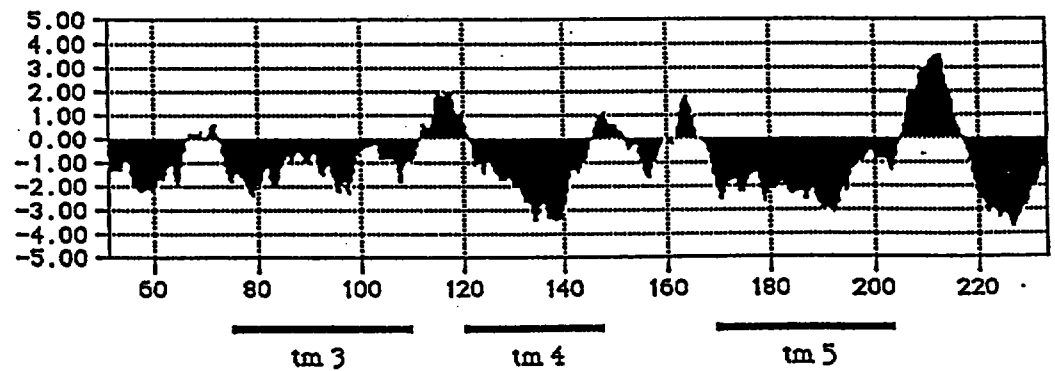


FIGURE 4

REPLACEMENT SHEET

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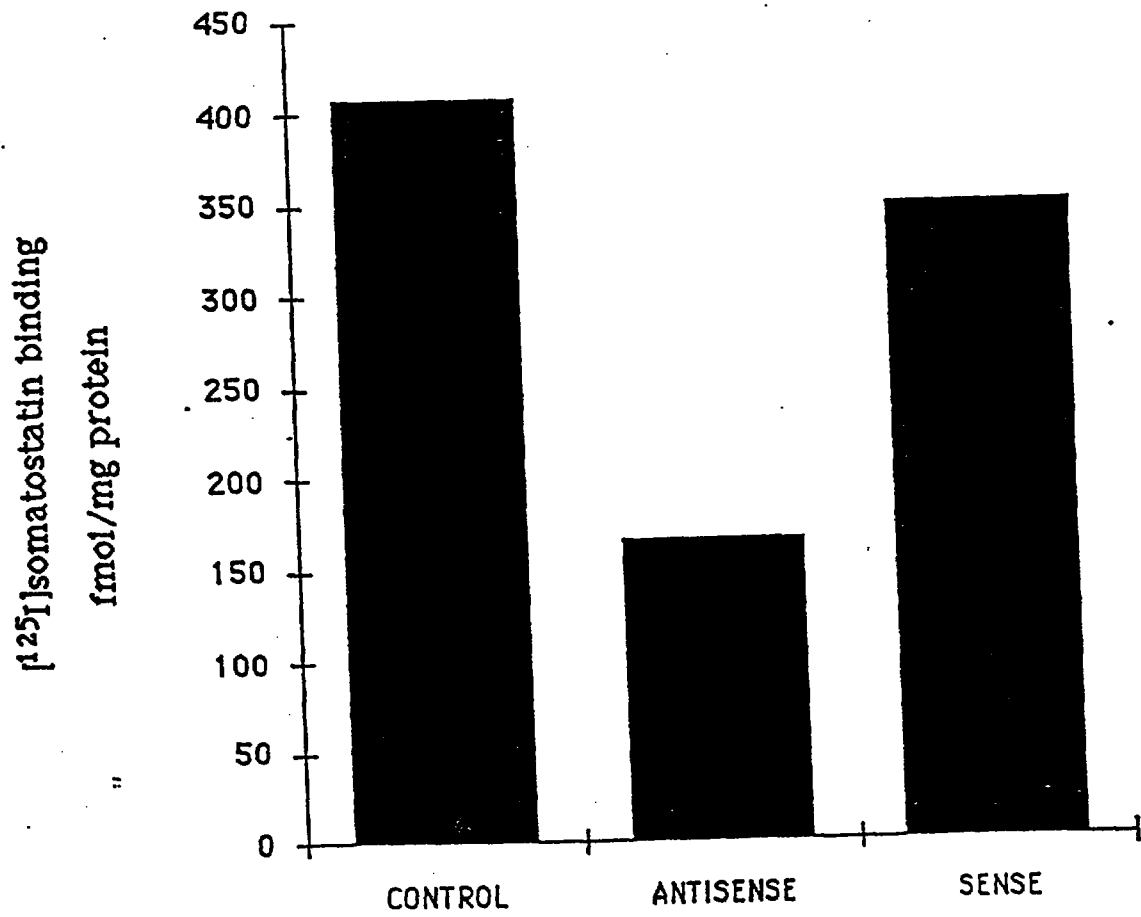
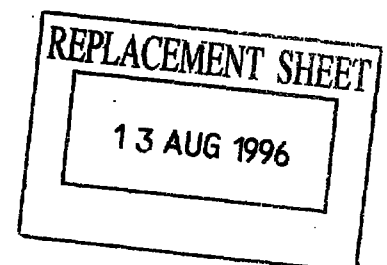
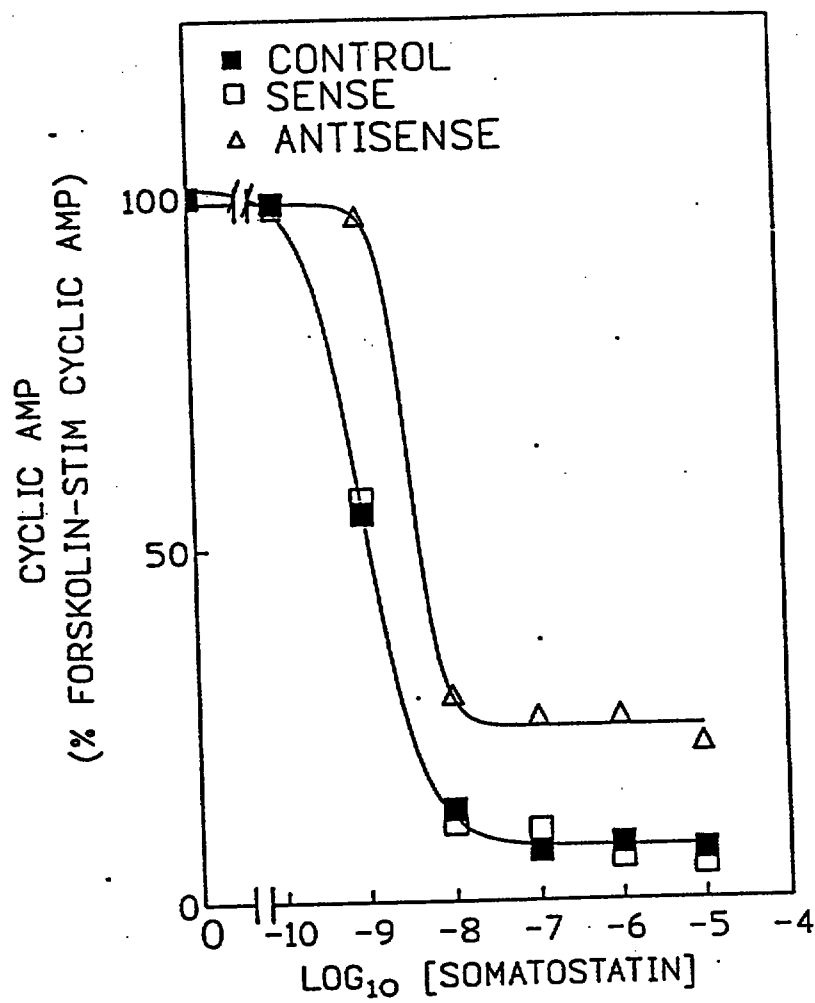


FIGURE 5





TREATMENT	ED ₅₀ (nM)
CONTROL	0.9
SENSE	1.1
ANTISENSE	7.2

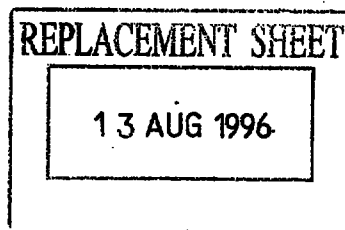
FIGURE 6

REPLACEMENT SHEET

13 AUG 1996

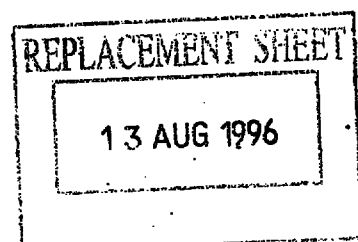
TTCGTGCTGA ACCTGGCCGG GGCTGACGTG TTGTTTATGT TGGGGCTTCC TTTCTTGCA 60
 ACGCAGAATG CTGTCTCCTA CTGGCCCTTT GGCTCCTTCT TGTGCCGCCT GGTCATGACG 120
 CTGGACGGCA TCAACCAGTT CACCAGTATC TTCTGCCTGA TGGTCATGAG TGTCGACCGC 180
 TACCTGGCCG TGGTCCACCC TCTCCGCTCA GCGCGGTGGC GTCGCCCACG GGTAGCCAAG 240
 CTGGCTAGTG CTGCCGTCTG GGTCTTCTCG CTGCTCATGT CTCTGCCGCT CTTGGTCTTT 300
 GCGGATGTCC AGGAGGGCTG GGGCAACTGC AACCTGAGCT GGCCAGAGCC TGTGGGAATG 360
 GGGGTGCAG CCTTCATCAC TTACACGTCT GTGCTGGGCT TCTTTGGGCC CCTGCTGGTC 420
 ATCTGCAATG GCTATTTGCT CATCGTAGTG AAGGTGAAGG CTGCAGGTAT GCGTGTGGGC 480
 TCCTCACGGC GGAGGCGCTC AGAACCCAAG GTGACTCGCA TGGTGGTGGT AGTGGTGCGG 540
 CTGTTCTGTG GCTGCTGGCT GCCTTTCTTC ATCGTCAACA TCGTCAACCT GGCCTTCACG 600
 CTACCCGAGG AGCCACCTC TGCCGGCCTC TACTTCTTG TGGTGGTCCT GTCTTATGCC 660
AATAGCCGCG CCAAGCCC

FIGURE 7



	2	3
SSTR3	VLFMLGLPFLATQNAVSYWPFGSFLCRLVMTLDGINQFTS	
2	E-----M-V-lVH---Kai--v---v-----	
1	E-l--Sv---V-STLlRH---Al-----lSv-Av-M---	
SSTR3	IFCLMVMSVDRYLAVVHPLRSARWRRPRVAKLASAAVWVF	
2	----T---i-----ik--k-----T--mINV---CV	
1	-y--T-l-----v-----ikA--Y---T---vVNLG---l	
	4	
SSTR3	SLLMSLPLLVFADVQEGWGNCNLSWPEPVGMWGAAFITYT	
2	---vI--imiy-GLRNQ--S-TiN--GES-A-YTG--I-A	
1	---vI--iv---ANSd-TVA--mLM---AQR-LVG-vL--	
	5	
SSTR3	SVLGFFGPLLVICMCYLLIVVKVKAAGMRVGSSRRRRSEP	
2	Fj---lV--Ti--l---f-ii---SS-i-----k-kk--K	
1	Flm--lL-vGA--l--v--iA-mrMValKA-WQQ-k---R	
	6	
SSTR3	KVTRMVVVVRLFVGCWLPFFIVNIVNLAFTLPEEPTSAG	
2	-----Si--Av-iF-----y-F-vSSvSVAiSPT-ALK-	
1	-i-L--mm--Mv--I--m--yv-Ql--v -AEQdda-VSQ	
	7	
SSTR3	LYFFVVVLSYA	
2	mfD---i-T--	
1	- S-i-G--	

FIGURE 8



cells and further avoids the necessity for having a full-length cDNA clone for heterologous expression in a host cell.

According to a further aspect of the invention there is provided an oligonucleotide selected from the group consisting of the oligonucleotides depicted in Figure 2A.

- 5 According to a further aspect of the invention there is provided an oligonucleotide selected from the group consisting of the oligonucleotides depicted in Figure 2B.

- 10 According to a further aspect of the invention there is provided an antisense oligonucleotide having the sequence GGGTCGCCTCCATTTCCG.

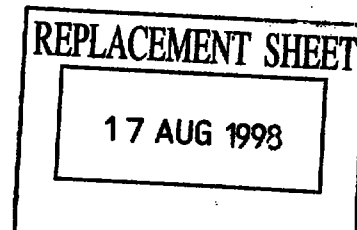
- 15 According to a further aspect of the invention there is provided an antisense oligonucleotide having the sequence CGAGCGCCTCCGCCTTGAGG.

- 20 According to a further aspect of the invention there is provided a nucleic acid fragment encoding a somatostatin receptor, comprising the amino acid sequence of SSTR3 depicted in Figure 3.

- 25 According to a further aspect of the invention there is provided a nucleic acid fragment encoding a somatostatin receptor, the fragment comprising the sequence depicted in Figure 7.

- 30 According to a further aspect of the invention there is provided a nucleic acid fragment encoding a somatostatin receptor, comprising the amino acid sequence of SSTR3 depicted in Figure 8.

- 35 According to a further aspect of the invention there is provided a nucleic acid fragment comprising the sequence of PCR9, depicted in Figure 3.



17 AUG 1998

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Procedure Summary. In this example, four orphan G protein-linked receptor gene fragments are isolated. Vehicle, sense (+; as a negative control) and antisense oligos (-) derived from each clone are tested in a cAMP accumulation assay to determine if one attenuates the response to a ligand known to activate adenylyl cyclase. In this case, an antisense oligo to orphan #3 represses the response, thereby identifying that sequence as a portion of the gene encoding the assayed receptor.

Figure 2. Description of oligonucleotides used as PCR primers. Lines with boxes represent genes encoding G protein-linked receptors. Each box indicates a transmembrane domain. Arrows correspond to binding sites for the described oligos. Nucleotide sequences use the IUPAC-IUB nomenclature for incompletely specified bases. A. Oligonucleotides directed toward the general class of peptide ligand receptors. B. Oligonucleotides directed toward the secretin receptor-like subclass.

Figure 3. Nucleotide sequences of orphan receptor DNA fragments. Underlined sequences indicate PCR primers. Both fragments contain oligo #8 at the 5' terminus and oligo #10 at the 3' terminus.

Figure 4. Hydropathy plots of peptides encoded by novel PCR fragments. The peptide product of each PCR fragment is analyzed by the Kyte-Doolittle hydropathy index. Each sequence begins within the second transmembrane domain and ends in the sixth transmembrane domain. Hydrophobic transmembrane domains 3, 4, and 5 are readily apparent. The plot