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**CAR RECEPTORS AND RELATED MOLECULES AND METHODS**
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- (57) Claim

1. Substantially pure CAR receptor polypeptide as herein defined.
2. The polypeptide of claim 1, comprising an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO:1).

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| <b>(54) Title:</b> CAR RECEPTORS AND RELATED MOLECULES AND METHODS<br><br><b>(57) Abstract</b><br><br>Purified DNA encoding CAR receptors and the recombinant proteins expressed from such DNA are disclosed. The recombinant receptor polypeptides are used to identify CAR ligands and CAR receptor binding sites and are also used to produce therapeutics. Antibodies specific for CAR receptor polypeptides are also disclosed.                                                                                                                                                                    |           |                                                                                                                                                                                                                                                                            |

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CAR RECEPTORS  
AND RELATED MOLECULES AND METHODS

Background of the Invention

5           This invention relates to receptors, particularly nuclear hormone receptors.

          In higher organisms, the nuclear hormone receptor superfamily includes approximately a dozen distinct genes that encode zinc finger transcription factors, each of  
10   which is specifically activated by binding a ligand such as a steroid, thyroid hormone (T3) or retinoic acid (RA). However, there is an additional, somewhat larger group of cDNAs that encode proteins that do not bind or respond to any known ligand. These members of the superfamily are  
15   called orphan receptors. While the role of the better characterized conventional receptors in regulating important processes in developing and adult individuals is becoming clearer, the function of the orphan receptors has been uncertain.

20           A number of the conventional and orphan members of the superfamily share identical or very similar amino acid sequences in an important region of the first zinc finger. Both genetic analyses and X-ray crystallography indicate that this region, termed the P box, makes  
25   sequence specific contacts with the DNA. The conventional receptors in this P box-defined subgroup include those that bind estrogen, vitamin D, T3 and RA, and nearly all of the orphan receptors identified to date also fall into this class. As a consequence of this  
30   overlap in binding specificity, many hormone response elements can bind more than one type of receptor. The best characterized of these is the element upstream of the rat growth hormone gene, which can be activated by three different isoforms of the T3 receptor encoded by  
35   two different genes and by an unknown number of retinoic

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acid receptor isoforms encoded by three different genes. While it does not appear to respond to the estrogen receptor or the vitamin D receptor, its response to other members of the subgroup remains uncertain.

5           Recently the potential complexity of the interactions of the conventional receptors with their response elements has been substantially increased by the demonstration that the three closely related RXR proteins can form heterodimers with the thyroid hormone, retinoic  
10 acid and vitamin D receptors. These heterodimers show higher binding affinity for appropriate response elements, and the RXRs are hypothesized to play central roles in signal transduction by all three classes of receptors. The impact on such heterodimers of the  
15 binding of the retinoid metabolite 9-cis retinoic acid by the RXRs remains unclear.

#### Summary of the Invention

In general, the invention features substantially pure CAR receptor polypeptide. Preferably, such a  
20 receptor polypeptide includes an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO:1).

The invention further features a substantially pure polypeptide which includes a CAR receptor DNA  
25 binding domain and a CAR receptor gene activation domain. Preferably, the DNA binding domain includes a sequence substantially identical to amino acids 11-75 of Fig. 1 (SEQ ID NO:1), or a DNA binding fragment thereof; and the gene activation domain includes a sequence substantially  
30 identical to amino acids 76-348 of Fig. 1 (SEQ ID NO:1), or a gene activating fragment thereof.

In a related aspect, the invention features a substantially pure polypeptide which includes a CAR receptor heterodimerization domain.

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In preferred embodiments of various aspects, the receptor polypeptide is mammalian, and preferably, human.

In yet other aspects, the invention features substantially pure DNA which encodes a CAR receptor polypeptide of the invention. Preferably, such DNA is cDNA; and encodes a human CAR receptor polypeptide. The invention also features a vector which includes such substantially pure DNA and which is capable of directing expression of the protein encoded by the DNA in a vector-containing cell. Finally, the invention features a cell which contains the substantially pure DNA. Preferably, the cell is a eukaryotic cell, for example, a mammalian cell.

In another aspect, the invention features a method of producing a recombinant CAR receptor polypeptide (or a fragment or analog thereof). The method involves (a) providing a cell transformed with DNA encoding a CAR receptor or a fragment or analog thereof positioned for expression in the cell; (b) culturing the transformed cell under conditions for expressing the DNA; and (c) isolating the recombinant CAR receptor polypeptide.

In yet another aspect, the invention features a substantially pure antibody which specifically binds a CAR receptor polypeptide of the invention.

In yet other aspects, the invention features compositions which include a CAR receptor polypeptide of the invention formulated in a physiologically-acceptable carrier; use of a CAR receptor polypeptide of the invention in the manufacture of a medicament for the treatment of Graves' disease in a mammal, the polypeptide being formulated in a dosage effective to decrease thyroid hormone receptor function; and use of a CAR receptor polypeptide of the invention in the manufacture of a medicament for the treatment of cancer in a mammal,

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the polypeptide being formulated in a dosage effective to increase retinoic acid receptor expression.

In yet other aspects, the invention features methods of identifying a CAR ligand. One method involves

5 (a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide; (b) introducing into a host cell which is functionally deficient for CAR receptor (i) the nucleic acid which encodes the CAR receptor polypeptide (preferably, a CAR receptor polypeptide including an

10 amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1; SEQ ID NO: 1) and (ii) a reporter gene operably linked to a CAR receptor polypeptide binding site (preferably, the binding site GGGTAGGGTTCACCGAAAGTTCACTCG; SEQ ID NO: 5); (c) measuring

15 induction of the reporter gene in the transfected host cell; (d) contacting the transfected host cell with a candidate ligand; and (e) measuring induction of said reporter gene in the presence of the candidate ligand, an increase or decrease in the induction as compared to the

20 induction in (c) being indicative of the presence of a CAR ligand.

The second method involves (a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide (preferably, including an amino acid sequence

25 substantially identical to the amino acid sequence shown in Fig. 1; SEQ ID NO: 1); (b) introducing the nucleic acid into a host cell so that the recombinant CAR receptor polypeptide is expressed; (c) isolating the recombinant protein; (d) immobilizing the recombinant

30 protein on a solid substrate (preferably, a column); (e) contacting the immobilized recombinant protein with a candidate ligand under conditions which allow formation of an affinity complex between the immobilized recombinant CAR receptor polypeptide and the candidate

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ligand; and (f) detecting complex formation as an indication of the presence of a CAR ligand.

In yet other aspects, the invention features methods of identifying a CAR receptor DNA binding site.

- 5 One method involves (a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide (preferably including an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1; SEQ ID NO: 1); (b) introducing into a host cell which is
- 10 functionally deficient for CAR receptor (i) the nucleic acid which encodes the CAR receptor polypeptide and (ii) a reporter gene which is operably linked to a candidate CAR receptor DNA binding site; and (c) measuring induction of the reporter gene in the transfected host
- 15 cell, induction being indicative of the presence of an operably linked CAR receptor DNA binding site.

- A second method involves (a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide (preferably, including an amino acid sequence
- 20 substantially identical to the amino acid sequence shown in Fig. 1; SEQ ID NO: 1); (b) introducing the nucleic acid into a host cell so that the recombinant CAR receptor polypeptide is expressed; (c) isolating the recombinant protein; (d) contacting the recombinant
- 25 protein with a candidate DNA binding site under conditions which allow formation of an affinity complex between the recombinant CAR receptor polypeptide and the candidate binding site; and (e) detecting complex formation as an indication of the presence of a CAR
- 30 receptor DNA binding site.

- In a final aspect, the invention features chimeric receptors. Such chimeric receptors may include the DNA binding domain of a CAR receptor polypeptide (preferably, including a sequence substantially identical to amino
- 35 acids 11-75 of Fig. 1; SEQ ID NO:1 or a DNA binding

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fragment thereof) fused to the gene activation (and, preferably, the ligand binding domain) of a heterologous protein, preferably, a nuclear hormone receptor, or a protein chosen from the group consisting of:

- 5 glucocorticoid receptor,  $\alpha$ -retinoic acid receptor,  $\beta$ -retinoic acid receptor,  $\gamma$ -retinoic acid receptor, estrogen receptor, progesterone receptor, vitamin D receptor, mineralocorticoid receptor, thyroid receptor, VP16, and GAL4; or the chimeric receptor may include the
- 10 gene activation domain of a CAR receptor polypeptide (preferably, including a sequence substantially identical to amino acids 76-348 of Fig. 1; SEQ ID NO:1, or a gene activating fragment thereof) fused to the DNA binding domain of a heterologous protein, preferably, a nuclear
- 15 hormone receptor or a protein chosen from the group consisting of: glucocorticoid receptor,  $\alpha$ -retinoic acid receptor,  $\beta$ -retinoic acid receptor,  $\gamma$ -retinoic acid receptor, estrogen receptor, progesterone receptor, vitamin D receptor, mineralocorticoid receptor, thyroid
- 20 receptor, and GAL4.

By "CAR receptor polypeptide" is meant a polypeptide which is capable of binding to a DNA sequence of GGGTAGGGTTCACCGAAAGTTCAC TCG (SEQ ID NO: 5) and activating the expression of downstream genes, even when

25 mammalian cells harboring the receptor are grown in medium containing charcoal-stripped serum; ~~in particular,~~ the CAR receptor polypeptide activates such gene expression in the absence of retinoic acid.

By "substantially pure" is meant that the CAR

30 receptor polypeptide provided by the invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most

35 preferably at least 99%, by weight, CAR receptor



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polypeptide. A substantially pure CAR receptor polypeptide may be obtained, for example, by extraction from a natural source (e.g., a mammalian liver cell); by expression of a recombinant nucleic acid encoding a CAR  
5 receptor polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. By a "polypeptide" is meant any chain of amino acids, regardless of length or  
10 post-translational modification (e.g., glycosylation)

By "substantially identical" is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine  
15 for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the protein or domain (assayed, e.g., as described herein). A "substantially  
20 identical" nucleic acid sequence codes for a substantially identical amino acid sequence as defined above.

By a "DNA binding domain" is meant a stretch of amino acids which is capable of directing specific  
25 polypeptide (e.g., receptor) binding to a particular DNA sequence.

By "gene activation domain" is meant a stretch of amino acids which is capable of inducing the expression of a gene to whose control region it is bound.

30 By "heterodimerization domain" is meant a stretch of amino acids which is capable of directing specific complex formation with a heterologous protein; such a domain may direct the formation of dimers, trimers, tetramers, or other higher order hetero-oligomers.

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By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore  
5 includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment  
10 produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which  
15 (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a CAR receptor polypeptide.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence  
20 which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., CAR receptor polypeptide).

By "a host cell which is functionally deficient for CAR receptor" is meant a cell, e.g., a mammalian  
25 cell, which exhibits little or no CAR receptor-mediated gene stimulatory activity; such activity may be measured in standard transactivation assays using, e.g., a transfected reporter gene operably linked to a CAR binding site as described herein.

30 By "substantially pure antibody" is meant antibody which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most  
35 preferably at least 99%, by weight, antibody, e.g., CAR

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receptor-specific antibody. A substantially pure CAR receptor antibody may be obtained, for example, by affinity chromatography using recombinantly-produced CAR receptor polypeptide and standard techniques.

5 By "specifically binds", as used herein, is meant an antibody which recognizes and binds CAR receptor polypeptide but which does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, which naturally includes CAR receptor  
10 polypeptide.

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, chloramphenicol transacetylase (CAT) and  $\beta$ -galactosidase.

15 By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

20 By "heterologous" is meant any protein other than the human CAR receptor which includes a suitable (i.e., a DNA binding, gene activation, and/or ligand binding) domain.

Other features and advantages of the invention  
25 will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

The drawings will first briefly be described.

#### Drawings

30 Fig. 1 is the nucleic acid sequence and deduced amino acid sequence of the human CAR receptor (SEQ ID NO:1).

There now follows a description of the cloning and characterization of a human CAR receptor-encoding cDNA

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useful in the invention. This example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

Isolation of the CAR Receptor

5 A human liver cDNA library was screened by standard techniques with the following degenerate oligonucleotide probe: TG C/T GAG GGI TG C/T AAG G G/C ITT C/T TT C/T A/C G (SEQ ID NO. 2). This probe was based on the sequence of the P box of the  
10 thyroid/retinoid receptor subgroup, the most highly conserved portion of the DNA binding domain. As expected, a number of clones encoding previously described members of the nuclear receptor superfamily were isolated. Based on limited sequence analysis (by  
15 standard techniques), one clone that did not correspond to any previously reported cDNA was chosen for further analysis. The complete sequence of this cDNA is presented in Fig. 1 (SEQ ID NO: 1).

As indicated in Fig. 1, this cDNA encodes a  
20 protein of 348 amino acids that contains conserved features of the nuclear receptor superfamily in both the DNA binding (C) domain and the putative ligand binding/dimerization (E) domain. Because of the activities of this protein described below, it is called  
25 CAR (Constitutive Activator of Retinoic acid response elements). CAR is one of the smallest superfamily members, with the shortest known A/B domain.

As expected from the oligonucleotide used for screening, the sequence of the P box DNA binding  
30 specificity determining region of the first zinc finger placed CAR in the thyroid/retinoid group. CAR was not strongly related to any other superfamily member, but was most similar to the vitamin D receptor, sharing 42 identical amino acids out of 66 in the C domain (64%),  
35 and 61/152 in the E domain (40%). This is quite similar

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to the relationship between the thyroid hormone and retinoic acid receptors: TR $\beta$  and RAR $\beta$  which share 60% and 40% sequence identity in the C and E domains, respectively. By comparison, the closely related human TR $\alpha$  and TR $\beta$  receptors share 90% and 85% identity in the C and E domain. CAR also shows significant similarity to the *Drosophila melanogaster* ecdysone receptor (62% in the C domain, 29% in the E domain), making it the third member of a divergent subgroup in the superfamily.

10 Southern blot analysis (by standard techniques) indicated that CAR may not be a member of a closely related subgroup like the TRs and the RARs.

Like a number of other members of the superfamily, CAR has a relatively long 5' untranslated region that contains several AUGs upstream of the start of the open reading frame. The function of this region is unknown.

Northern blot analysis (using standard techniques) indicated that CAR mRNA is expressed in a variety of human tissues, but is most abundant in liver. Multiple RNA species were observed, but the nature of these different species is not clear. Based on the multiple products expressed by genes encoding other superfamily members, it may well reflect alternative splicing or promoter utilization. CAR mRNA is also observed in a number of human cell lines, including HepG2 (hepatoma), JEG-3 (choriocarcinoma), and HeLa.

Ligand-independent transcriptional activation by the CAR ligand/dimerization domain

To study the function of the putative ligand binding domain of the orphan, a chimeric receptor consisting of the C-terminal D, E and F domains of CAR (i.e., approximately amino acids 76-172, 173-319, and 320-348 of the CAR sequence, respectively) fused to the N-terminal A/B and C domains of TR $\beta$  was generated (termed TR/CAR), along with a control hybrid with the A/B and C

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domains of TR and the D, E and F domain of the glucocorticoid receptor (TR/GR). Vectors expressing these chimeras or the intact TR were cotransfected into JEG3 cells (ATCC Accession No: HTB 36) with reporter  
5 plasmids containing T3REs (i.e., of sequence AAAGGTAAGATCAGGGACGTGACCGCAG; SEQ ID NO: 3) or GREs (from the MMTV promoter as described in Chandler et al., *Cell* 33:489, 1989) upstream of a reporter gene whose expression could be assayed. Transfections were carried  
10 out in the presence of serum treated with activated charcoal to remove T3 as well as other low molecular weight hydrophobic compounds that could act as CAR ligands.

Results from such an analysis revealed that both  
15 TR and the TR/GR chimera behaved as ligand-dependent transactivators, as expected. The relatively low but reproducible level of activation conferred by TR/GR is consistent with a previous report, and is thought to be a consequence of the absence from this hybrid of  
20 transcriptional activation domains present in the A/B domain of the intact GR and the F domain of the intact TR.

In contrast, the TR/CAR chimera activated expression of the T3RE containing reporter in the absence  
25 of any added ligand. This effect was not altered by addition of T3, estradiol, testosterone, retinoic acid, or dexamethasone, or by the addition of the orphan receptor ligand 25-hydroxy cholesterol. The constitutive activation conferred by this hybrid was substantial,  
30 corresponding to greater than 50% of the response of the intact TR $\beta$ . Similar results were observed with an analogous GR/CAR chimera in cotransfections with an MMTV/CAT reporter.

The activation conferred by the TR/CAR chimera in  
35 the presence of charcoal-rippled serum could be a

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consequence of either a direct, constitutive transcriptional activation function in the D, E or F domains of the orphan, or the presence of an uncharacterized ligand in the medium of the growing  
5 cells. To minimize the possibility of the presence of such a ligand in the media, the transfections were repeated in the absence of serum. Such conditions substantially reduced the level of both control and T3-activated expression, but did not prevent the  
10 constitutive activation function conferred by the TR/CAR chimera. Because of the substantial changes in the levels of expression from control promoters, the significance of the apparent decrease in constitutive activity relative to the level of activation conferred by  
15 TR $\beta$  in the presence of hormone is uncertain. This apparent decrease may reflect the absence of some specific stimulator of CAR function present in serum or could be a less specific effect associated with the unfavorable growth conditions.

20 Activation of retinoic acid response elements by CAR

Based on the constitutive activity of the TR/CAR chimera and the similarity of the DNA binding domain of CAR to other members of the superfamily, a number of response elements were screened for activation by the  
25 intact orphan in standard cotransfections. Despite the relatively close sequence relationship between CAR and the vitamin D receptor, no response was seen with the combined vitamin D/RA response element from the rat osteocalcin gene (i.e., of sequence  
30 TGGGTGAATGAGGACATTACTGACCGCTCCG; SEQ ID NO: 4). However, CAR did transactivate two wild type elements, the RAREs from the RAR $\beta$  gene and the alcohol dehydrogenase (ADH) gene (i.e., GGGTAGGGTTCACCGAAAGTTCACCTCG; SEQ ID NO: 5). RAREs which were not transactivated by CAR included a  
35 potent up mutant version of the rat growth hormone gene

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T3RE/RARE (i.e., of sequence  
AAAGGTAAGATCAGGGACGTGACCTCAG; SEQ ID NO:6; in tandem  
copies with the second copy inverted; described in Brent  
et al., *Mol. Endocrinol.* 3:1996, 1989) and the wild type  
5 version of the laminin T3RE/RARE (i.e., of sequence  
AGACAGGTTGACCCTTTTTCTAAGGGCTTAACCTAGCTCACCTG; SEQ ID NO:  
8). The rat malic enzyme T3RE (i.e., of sequence  
AGGACGTTGGGGTTAGGGGAGGACAGTG; SEQ ID NO: 9) and the rat  
 $\alpha$ -myosin heavy chain T3RE (i.e., of sequence  
10 CTGGAGGTGACAGGAGGACAGCAGCCCTGA; SEQ ID NO: 7), which do  
not respond to RARs, did not respond to CAR.

The activation of the RAR $\beta$  element by CAR is not  
affected by several treatments that activate or  
inactivate signal transduction pathways mediated by  
15 protein kinases A or C. Thus, CAR activation was not  
altered by addition of dibutyryl cyclic AMP, by short or  
long term treatments with phorbol esters, or by  
cotransfections with vectors expressing the specific  
protein kinase A inhibitor peptide.

20 The activation of the RAREs by CAR suggests that  
this orphan could play an important role in the complex  
regulatory network that controls expression of RA  
responsive genes. To examine the functional interactions  
between CAR and RAR $\beta$ , expression vectors for both were  
25 mixed in cotransfections. Increasing amounts of the CAR  
expression vector were added to a  $\beta$ RARE (SEQ ID NO: 5)-  
containing CAT reporter plasmid and a fixed amount of  
RAR $\beta$  expression vector corresponding to approximately 2/3  
of the level that is saturating for induction. In the  
30 absence of RA, the increasing amount of CAR led to an  
increase in basal expression. In the presence of RA, the  
high level of activated expression was not strongly  
affected by addition of CAR. Together, these effects  
resulted in a significant decrease in the RA induction  
35 ratio with increasing amounts of CAR.

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These results indicate that the two superfamily members function independently at this response element. Analogous results were obtained when increasing amounts of RAR $\beta$  were added to a subsaturating amount of CAR expression vector. In the absence of RA, the constitutive activation conferred by CAR could be blocked by excess RAR $\beta$ . This is consistent with previous reports that RARs can repress expression in the absence of ligand. When retinoic acid was added, activation was observed at even moderate levels of RAR $\beta$ . The levels of activated expression associated with the various doses of RAR $\beta$  vector were similar to those observed in the absence of cotransfected CAR. It therefore seems most likely that CAR and RAR $\beta$  do not interfere with each other when co-expressed at moderate levels. Under other circumstances, more complex indirect effects could be anticipated for the interaction between CAR and RXR (see below).

In contrast, addition of low levels of RXR $\alpha$  stimulated the effect of a subsaturating dose of CAR expression vector. Since similar results are observed with the receptors able to heterodimerize with RXRs, this result strongly suggests that RXR $\alpha$  may share a similar interaction with CAR.

#### 25 DNA binding by CAR

To confirm that CAR binds the  $\beta$ RARE, nuclear extracts from HeLa cells infected with vaccinia virus vectors overexpressing FLAG epitope-tagged versions of CAR or RAR $\alpha$  were used in standard gel shift experiments. Both CAR- and RAR $\alpha$ -containing extracts showed specific binding to the RAR $\beta$  element (SEQ ID NO: 5), with RAR binding being of higher apparent affinity. In agreement with results of cotransfections, CAR binding was strongly stimulated by addition of nuclear extract from vaccinia infected HeLa cells overexpressing RXR $\alpha$ . However, little

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or no effect on affinity was observed when CAR and RAR were mixed. No evidence for formation of CAR/RAR heterodimers was observed in gels electrophoresed for longer times to better resolve the CAR and RAR shifted  
5 complexes. From these results, it appears that CAR can bind directly to the RAR $\beta$  element, and that binding is strongly stimulated by RXR $\alpha$ .

The constitutive activity of CAR may be due to either a truly constitutive transcriptional activation  
10 function or the presence of some ubiquitous ligand. The former possibility is supported by its activity in serum free media and in distinct cell types. The existence of a ligand may be favored by the somewhat lower relative activity observed with serum free medium compared to  
15 medium containing charcoal stripped serum. However, the substantial changes in the expression of the control and activated promoters under these two quite distinct growth conditions suggest that less specific or direct effects could explain this difference.

20 Negative results have been obtained by several approaches designed to determine whether the constitutive activation of CAR is associated with various second messenger pathways. Evidence obtained to date indicates that CAR function is not affected by activation or  
25 repression of the activity of protein kinases A or C.

Based on the results presented here, it is possible that CAR plays two important roles in the complex, interlocking set of proteins that determines responses to RA, T3 and vitamin D. The first is to  
30 maintain a basal level of expression of a subset of RA responsive genes in the absence of the ligand. In the case of a cell expressing only RAR $\beta$ , for example, this could allow expression of sufficient levels of the receptor to allow autoactivation of the RA-dependent  
35 positive feedback loop that regulates RAR $\beta$  expression

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upon addition of ligand. The second potential function, is based on the interaction of CAR with RXR. Increasing expression of CAR would be expected to decrease the amount of RXR available for interaction with other  
5 heterodimeric partners. Thus, in a cell with limiting amounts of RXR, alterations in the amount or activity of CAR protein could have significant effects on the activity of RARs, T3Rs or VDR. Although the levels of CAR used in the cotransfections reported here did not  
10 show an antagonistic effect on RAR activity, preliminary results indicate that inhibitory effects of this type can be observed in other circumstances. Given the remarkable complexity of the regulatory networks that control response to the retinoids and the other ligands of this  
15 subgroup of the nuclear receptor superfamily, it is likely that even more complicated functions will be found for CAR.

#### Polypeptide Expression

Polypeptides according to the invention may be  
20 produced by transformation of a suitable host cell with all or part of a CAR receptor-encoding cDNA fragment (e.g., the cDNA described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology  
25 will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise host cell used is not critical to the invention. The CAR receptor may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host  
30 (e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g., COS 1, NIH 3T3, and JEG3 cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *Current Protocols in Molecular*  
35 *Biology*, John Wiley & Sons, New York, 1989). The method

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of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987).

One preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides: an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding a CAR receptor polypeptide would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant receptor protein would be isolated as described below. Other preferable host cells which may be used in conjunction with the pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

Alternatively, a CAR receptor polypeptide is produced by a stably-transfected mammalian cell line.

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (*supra*). In one example, cDNA encoding the receptor polypeptide is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the CAR receptor-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300

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- $\mu$ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated
- 5 amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate.
- 10 DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdd26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR<sup>-</sup>cells, ATCC Accession No. CRL 9096)
- 15 are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

- Once the recombinant CAR receptor polypeptide is expressed, it is isolated, e.g., using affinity
- 20 chromatography. In one example, a CAR binding site (e.g., the  $\beta$ RARE site described above) or an anti-CAR receptor antibody (e.g., produced as described below) may be attached to a column and used to isolate the receptor polypeptide. Lysis and fractionation of receptor-
- 25 harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, *Laboratory*
- 30 *Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Receptors of the invention, particularly short receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase*

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Peptide Synthesis, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate  
5 useful CAR receptor fragments or analogs (described below). Identification of Ligands which Bind CAR Receptors

Although the CAR receptor described above was capable of activating some level of target gene  
10 expression in the apparent absence of a ligand, this does not discount the possibility that the receptor interacts with one or more ligands, e.g., to modulate receptor activity. Moreover, it is possible that multiple CAR  
15 receptors exist (e.g., as the products of a differentially-spliced CAR mRNA) and that different receptor species interact with different ligands. Accordingly, one aspect of the invention features a screening assay for the identification of compounds which specifically bind to the CAR receptors described herein.  
20 Such an assay may be carried out using a recombinant receptor protein.

In one example, the CAR receptor component is produced by a cell that naturally produces substantially no receptor or by a cell which produces functionally  
25 deficient receptor (i.e., a cell which apparently expresses CAR receptor mRNA as measured by Northern blotting but which does exhibit reporter gene induction, in the absence of recombinantly-produced CAR receptor, in a transactivation assay, see below); suitable cells are,  
30 e.g., those discussed above with respect to the production of recombinant receptor, most preferably, mammalian cells such as JEG3 cells. Host cells are transfected with (1) a vector which expresses a nucleic acid encoding the CAR receptor component (i.e., the  
35 "producer vector") and (2) a vector which includes a CAR

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receptor binding site (e.g., the  $\beta$ RARE sequence GGGTAGGGTTTCACCGAAAGTTCACTCG; SEQ ID NO: 5; described above) positioned upstream of a target gene which may be assayed (e.g., a CAT gene or a  $\beta$ -galactosidase gene) 5 (i.e., the "reporter vector"). Using such a standard transactivation assay procedure, CAR receptor activity is assayed by measuring CAR binding site-dependent target gene expression. CAR ligands are identified as those compounds which, when added to the host cell medium, 10 effect a change in CAR receptor-directed gene expression (as detected using any CAR reporter vector); a CAR ligand according to the invention may either increase CAR receptor activity or decrease CAR receptor activity.

Any suitable transactivation technique, CAR 15 receptor-encoding producer vector, and CAR receptor binding site-containing reporter vector may be used. Descriptions of transactivation assays and generally useful vectors for the identification of ligands which bind other nuclear hormone receptors are described, e.g., 20 in Evans et al. (U.S. Pat. No. 4,981,784, 1991); Evans et al. (WO 90/07517); Evans et al. (WO90/01428); and WO88/03168; all hereby incorporated by reference. CAR receptor polypeptides which may be used to screen for CAR ligands include wild-type molecules as well as any 25 appropriate chimeric receptor, for example, the GR/CAR and TR/CAR receptors described above.

Candidate ligands may be purified (or substantially purified) molecules or the ligand may be one component of a mixture of ligands (e.g., an extract 30 or supernatant obtained from cells; Ausubel et al., supra). In a mixed ligand assay, the CAR ligand is identified by testing progressively smaller subsets of the ligand pool (e.g., produced by standard purification techniques, e.g., HPLC or FPLC) until a single ligand is 35 finally demonstrated to modulate the CAR receptor gene

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stimulatory activity. Candidate CAR ligands include peptide as well as non-peptide molecules.

Alternatively, a ligand may be identified by its ability to bind a CAR receptor polypeptide using affinity  
5 chromatography. Recombinant receptor is purified by standard techniques, from cells engineered to express the receptor (e.g., those described above); the recombinant receptor immobilized on a column (e.g., a Sepharose column or a streptavidin-agarose column by the  
10 immunoaffinity method of Ausubel et al., supra) and a solution containing one or more candidate ligands is passed through the column. Such a solution (i.e., such a source of candidate ligands) may be, e.g., a cell extract, mammalian serum, or growth medium on which  
15 mammalian cells have been cultured and into which the cells have secreted factors (e.g., growth factors) during culture; again, candidate CAR ligands include peptide as well as non-peptide molecules. A ligand specific for a recombinant receptor is immobilized on the column  
20 (because of its interaction with the receptor). To isolate the ligand, the column is first washed to remove non-specifically bound molecules, and the ligand of interest is then released from the column and collected.

CAR ligands isolated by the above methods (or any  
25 other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography; see above). Once isolated in sufficiently-purified form, a novel peptide ligand may be partially sequenced (by standard amino acid sequencing  
30 techniques). From this partial amino acid sequence, a partial nucleic acid sequence is deduced which allows the preparation of primers for PCR cloning of the ligand gene (e.g., by the method of Ausubel et al., supra).

Identification of CAR Receptor DNA Binding Sites

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Identification of the CAR receptor facilitates identification of its DNA binding site(s). According to one approach, CAR receptor DNA binding sites may be identified using a transactivation assay, e.g., as  
5 described above for the identification of the binding site of sequence GGGTAGGGTTCACCGAAAGTTCACTCG (SEQ ID NO: 5). Briefly, candidate DNA binding sites are inserted upstream of a target gene (whose expression may be assayed, e.g., those genes described above) and the  
10 ability of a CAR receptor polypeptide to bind the DNA site is assayed as its ability to activate downstream gene expression.

Alternatively, a DNA binding site may be identified by selectively retaining a receptor-bound DNA  
15 fragment on a nitrocellulose filter. This approach relies on the ability of nitrocellulose to bind proteins but not double-stranded DNA. Purified CAR receptor polypeptide (e.g., purified by standard techniques from cells engineered to express the receptor, e.g., those  
20 described above) is mixed with labelled double-stranded DNA (e.g., a random pool of DNA fragments) under conditions which allow interaction. After incubation, the mixture is suction filtered through nitrocellulose, allowing unbound DNA to pass through the filter while  
25 retaining the protein and any DNA specifically bound to it. Bound DNA fragments are then eluted from the filter and analyzed by gel electrophoresis or amplification and cloning. A detailed description of this technique is published in Ausubel et al., supra).

30 Candidate DNA fragments for either approach may be derived from a randomly cleaved or sonicated genomic DNA library and/or may be derived from known nuclear hormone response elements (see, e.g., Evans et al., WO90/11273).

Identification of CAR receptor DNA binding sites  
35 facilitates a search for the presence of such sites

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upstream of known or yet unidentified genes (e.g., by an examination of sequences upstream of known genes or by standard hybridization screening of a genomic library with binding site probes). CAR-mediated transcriptional control of genes bearing the binding site upstream may then be investigated (e.g., by transactivation experiments as described above), potentially leading to the elucidation of novel CAR receptor functions.

#### Chimeric Receptors

10           The functional domains of the CAR receptor may be swapped with the domains of other members of the nuclear hormone receptor family (see, e.g., Evans et al., WO 90/11273; Evans, *Science* 240:889, 1988) in order to produce receptors having novel properties. For example, 15 if the DNA binding domain of the glucocorticoid receptor were fused to the gene activation domain of the CAR receptor, a novel receptor would be produced which could bind genes bearing an upstream glucocorticoid response element and activate gene expression in the absence of 20 hormone. Conversely, fusion of the CAR DNA binding domain to the ligand-binding and gene activation domains of glucocorticoid receptor would confer hormonal regulation on genes downstream of CAR binding sites. Finally, fusion of the CAR DNA binding domain to a trans- 25 repressing domain (see, e.g., Evans et al., WO90/14356) would result in repression of the basal level of expression of genes bearing upstream CAR binding sites. Construction of receptor fusion genes is carried out by standard techniques of molecular biology. CAR receptor 30 domains are as follows: DNA binding domain, approximately amino acids 11-76; and gene activation and potential ligand binding domain, approximately amino acids 76-348. Examples of receptor domains which may be included in a chimeric CAR receptor are described in Evans et al. (WO 35 90/15815) and in Evans et al. (*Science* 240:889, 1988).

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Dominant Negative Mutants

Mutants of the CAR receptor may be generated which interfere with normal CAR receptor activity. Such mutants are termed "dominant negative" and fall into at least two classes: (a) ones which bind to their DNA binding site (thereby interfering with the ability of wild-type receptor to bind the same site) and which do not activate gene expression and (b) ones which heterodimerize with other receptors (e.g., RXR) but which do not promote the biological response associated with the wild-type heterodimer.

The first class of CAR dominant negative mutants include those receptor polypeptides which contain a wild-type DNA binding domain and a mutant gene activation domain. Such mutants are unable to transactivate a reporter gene (e.g., as measured using a CAT reporter gene with an upstream  $\beta$ RARE and the standard methods described above) but retain the ability to bind a CAR DNA binding site (as evidenced, e.g., by DNA footprint analysis using a  $\beta$ RARE DNA sequence; Ausubel et al., supra).

The second class of CAR dominant negative mutants include those receptor polypeptides which contain a wild-type heterodimerization domain. Such a mutant interacts with its heterodimer partner and disrupts the partner's function. In one particular example, a dominant negative CAR receptor polypeptide may be overproduced (e.g., by directing its expression from a very strong promoter); the abundant CAR receptor polypeptide forms heterodimers with cellular RXR protein, soaking up available RXR and thereby preventing RXR homodimer formation as well as RXR heterodimer formation with other partner proteins (e.g., RAR, VDR, and TR). Wild-type CAR receptor polypeptide may function as a dominant negative mutant if overproduced in this manner. However, a mutant CAR

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receptor lacking a gene activation domain (e.g., as identified above) and/or a DNA binding domain (e.g., as identified by DNA footprint analysis, above) is preferred.

- 5 Any of the above mutants may be generated by any method of random or site-directed DNA mutagenesis (see, e.g., Ausubel et al., supra).

Identification of Molecules which Modulate CAR Receptor Expression

- 10 Isolation of the CAR receptor gene also facilitates the identification of molecules which increase or decrease CAR receptor expression, and which may be useful as therapeutics, e.g., for treatment of cancers such as lung cancer, or for treatment of thyroid  
15 disorders such as Graves' disease. According to one approach, candidate molecules (e.g., peptide or non-peptide molecules found, e.g., in a cell extract, mammalian serum, or growth medium on which mammalian cells have been cultured) are added at varying  
20 concentrations to the culture medium of cells which express CAR receptor mRNA (e.g., HepG2, JEG-3, or HeLa cells). CAR receptor expression is then measured by standard Northern blot analysis (Ausubel et al., supra) using CAR receptor cDNA as a hybridization probe. The  
25 level of CAR receptor expression in the presence of the candidate molecule is compared to the level measured for the same cells in the same culture medium but in the absence of the candidate molecule. A molecule which promotes an increase or decrease in CAR receptor  
30 expression is considered useful in the invention.

Anti-CAR Receptor Antibodies

- Human CAR receptor (or immunogenic receptor fragments or analogues) may be used to raise antibodies useful in the invention; such polypeptides may be  
35 produced by recombinant or peptide synthetic techniques

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(see, e.g., *Solid Phase Peptide Synthesis*, supra; Ausubel et al., supra). The peptides may be coupled to a carrier protein, such as KLH as described in Ausubel et al, supra. The KLH-peptide is mixed with Freund's adjuvant  
5 and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by peptide antigen affinity chromatography.

Monoclonal antibodies may be prepared using the CAR polypeptides described above and standard hybridoma  
10 technology (see, e.g., Kohler et al., *Nature* 256:495, 1975; Kohler et al., *Eur. J. Immunol.* 6:511, 1976; Kohler et al., *Eur. J. Immunol.* 6:292, 1976; Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, NY, 1981; Ausubel et al., supra).

15 Once produced, polyclonal or monoclonal antibodies are tested for specific CAR receptor recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., supra). Antibodies which specifically recognize a CAR receptor polypeptide  
20 are considered to be useful in the invention; such antibodies may be used, e.g., in an immunoassay to monitor the level of CAR receptor produced by a mammal.

#### Therapy

Because a lack of retinoic acid receptor has been  
25 associated with the occurrence of lung cancer and because the CAR receptor polypeptide binds and activates expression of the retinoic acid receptor gene, it is likely that the administration of a CAR receptor polypeptide to a mammal may prevent or treat cancers,  
30 particularly lung cancer. Similar therapeutic results would be expected for administration of a ligand which stimulates CAR receptor activity.

CAR receptor polypeptides may also find therapeutic use in the treatment of Graves disease, a  
35 disease resulting from an increase in thyroid hormone

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receptor function. RXR protein plays a role in thyroid hormone receptor expression. Accordingly, dominant negative CAR mutants which heterodimerize with RXR protein (including overexpressed wild-type CAR receptor protein) may act to decrease the cellular levels of available RXR and thereby decrease thyroid hormone receptor function. Again, ligands which increase heterodimerization efficiency could also be administered as a treatment for Graves' disease.

10 To treat the above diseases, the appropriate CAR receptor polypeptide (or ligand) is administered as a therapeutic preparation (e.g., in physiological saline) in accordance with the condition to be treated. Ordinarily, it will be administered intravenously, at a dosage effective to increase retinoic acid receptor expression (as a cancer treatment) or effective to decrease thyroid hormone receptor function (as a treatment for Graves' disease). Alternatively, it may be convenient to administer the therapeutic orally, nasally, 15 or topically, e.g., as a liquid or a spray. Again, the dosages are as described above. Treatment may be repeated as necessary for alleviation of disease symptoms.

The methods of the invention may be used to reduce 25 the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated, the CAR receptor polypeptide or the antibody employed is preferably specific for that species.

### 30 Other Embodiments

Polypeptides according to the invention include the entire human CAR receptor (as described in Fig. 1; SEQ ID NO:1) as well as any analog or fragment of the human CAR receptor which includes either a DNA binding 35 domain and a gene activation domain; or which includes a

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heterodimerization domain (as identified using the techniques described above).

Polypeptides of the invention also include all mRNA processing variants (e.g., all products of  
5 alternative splicing or differential promoter utilization) as well as CAR receptor proteins from other mammals.

Specific receptor fragments or analogues of interest include full-length or partial (see below)  
10 receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino  
15 acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to either bind DNA and activate transcription; or to interact with CAR receptor's heterodimerization partners (as assayed above). Analogs  
20 also include receptor polypeptides which are modified for the purpose of increasing peptide stability; such analogs may contain, e.g., one or more desaturated peptide bonds or D-amino acids in the peptide sequence or the peptide may be formulated as a cyclized peptide molecule.

25 Other embodiments are within the following claims.

- 30 -

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: David D. Moore et al.
- (ii) TITLE OF INVENTION: CAR RECEPTORS AND RELATED MOLECULES AND METHODS
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:

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(C) CITY: Boston  
(D) STATE: Massachusetts  
(E) COUNTRY: U.S.A.  
(F) ZIP: 02110-2804

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb  
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX  
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)  
(D) SOFTWARE: WordPerfect (Version 5.0)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/843,350  
(B) FILING DATE: February 26, 1992

## (viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 30,162  
(C) REFERENCE/DOCKET NUMBER: 00786/126001

## (ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (617) 542-8906  
(C) TELEX: 200154

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1450  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

- 31 -

|                                                                                                                                                       |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| GTGAGCTTGC TCCTTAAGTT ACAGGAACTC TCCTTATAAT AGACACTTCA TTTTCCTAGT                                                                                     | 60  |
| CCATCCCCTCA TGAAAAATGA CTGACCACTG CTGGGCAGCA GGAGGGATGA TAATCCTAAC                                                                                    | 120 |
| TCCAATCACT GGCAACTCCT GAGATCAGAG GAAAACCAGC AACAGCGTGG GAGTTTGGGG                                                                                     | 180 |
| AGAGGCATTC CATACCAGAT TCTGTGGCCT GCAGGTGACA TGCTGCCTAA GAGAAGCAGG                                                                                     | 240 |
| AGTCTGTGAC AGCCACCCCA ACACGTGACG TC                                                                                                                   | 272 |
| ATG GCC AGT AGG GAA GAT GAG CTG AGG AAC TGT GTG GTA TGT GGG GAC<br>Met Ala Ser Arg Glu Asp Glu Leu Arg Asn Cys Val Val Cys Gly Asp<br>1 5 10 15       | 320 |
| CAA GCC ACA GGC TAC CAC TTT AAT GCG CTG ACT TGT GAG GGC TGC AAG<br>Gln Ala Thr Gly Tyr His Phe Asn Ala Leu Thr Cys Glu Gly Cys Lys<br>20 25 30        | 368 |
| GGT TTC TTC AGG AGA ACA GTC AGC AAA AGC ATT GGT CCC ACC TGC CCC<br>Gly Phe Phe Arg Arg Thr Val Ser Lys Ser Ile Gly Pro Thr Cys Pro<br>35 40 45        | 416 |
| TTT GCT GGA AGC TGT GAA GTC AGC AAG ACT CAG AGG CGC CAC TGC CCA<br>Phe Ala Gly Ser Cys Glu Val Ser Lys Thr Gln Arg Arg His Cys Pro<br>50 55 60        | 464 |
| GCC TGC AGG TTG CAG AAG TGC TTA GAT GCT GGC ATG AGG AAA GAC ATG<br>Ala Cys Arg Leu Gln Lys Cys Leu Asp Ala Gly Met Arg Lys Asp Met<br>65 70 75 80     | 512 |
| ATA CTG TCG GCA GAA GCC CTG GCA TTG CGG CGA GCA AAG CAG GCC CAG<br>Ile Leu Ser Ala Glu Ala Leu Ala Leu Arg Arg Ala Lys Gln Ala Gln<br>85 90 95        | 560 |
| CGG CGG GCA CAG CAA ACA CCT GTG CAA CTG AGT AAG GAG CAA GAA GAG<br>Arg Arg Ala Gln Gln Thr Pro Val Gln Leu Ser Lys Glu Gln Glu Glu<br>100 105 110     | 608 |
| CTG ATC CGG ACA CTC CTG GGG GCC CAC ACC CGC CAC ATG GGC ACC ATG<br>Leu Ile Arg Thr Leu Leu Gly Ala His Thr Arg His Met Gly Thr Met<br>115 120 125     | 656 |
| TTT GAA CAG TTT GTG CAG TTT AGG CCT CCA GCT CAT CTG TTC ATC CAT<br>Phe Glu Gln Phe Val Gln Phe Arg Pro Pro Ala His Leu Phe Ile His<br>130 135 140     | 704 |
| CAC CAG CCC TTG CCC ACC CTG GCC CCT GTG CTG CCT CTG GTC ACA CAC<br>His Gln Pro Leu Pro Thr Leu Ala Pro Val Leu Pro Leu Val Thr His<br>145 150 155 160 | 752 |
| TTC GCA GAC ATC AAC ACT TTC ATG GTA CTG CAA GTC ATC AAG TTT ACT<br>Phe Ala Asp Ile Asn Thr Phe Met Val Leu Gln Val Ile Lys Phe Thr<br>165 170 175     | 800 |
| AAG GAC CTG CCC GTC TTC CGT TCC CTG CCC ATT GAA GAC CAG ATC TCC<br>Lys Asp Leu Pro Val Phe Arg Ser Leu Pro Ile Glu Asp Gln Ile Ser<br>180 185 190     | 848 |
| CTT CTC AAG GGA GCA GCT GTG GAA ATC TGT CAC ATC GTA CTC AAT ACC<br>Leu Leu Lys Gly Ala Ala Val Glu Ile Cys His Ile Val Leu Asn Thr<br>195 200 205     | 896 |

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|                                                                                                                                                       |      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| ACT TTC TGT CTC CAA ACA CAA AAC TTC CTC TGC GGG CCT CTT CGC TAC<br>Thr Phe Cys Leu Gln Thr Gln Asn Phe Leu Cys Gly Pro Leu Arg Tyr<br>210 215 220     | 944  |
| ACA ATT GAA GAT GGA GCC CGT GTG GGG TTC CAG GTA GAG TTT TTG GAG<br>Thr Ile Glu Asp Gly Ala Arg Val Gly Phe Gln Val Glu Phe Leu Glu<br>225 230 235 240 | 992  |
| TTG CTC TTT CAC TTC CAT GGA ACA CTA CGA AAA CTG CAG CTC CAA GAG<br>Leu Leu Phe His Phe His Gly Thr Leu Arg Lys Leu Gln Leu Gln Glu<br>245 250 255     | 1040 |
| CCT GAG TAT GTG CTC TTG GCT GCC ATG GCC CTG TTC TCT CCT GAC CGA<br>Pro Glu Tyr Val Leu Leu Ala Ala Met Ala Leu Phe Ser Pro Asp Arg<br>260 265 270     | 1088 |
| CCT GGA GTT ACC CAG AGA GAT GAG ATT GAT CAG CTG CAA GAG GAG ATG<br>Pro Gly Val Thr Gln Arg Asp Glu Ile Asp Gln Leu Gln Glu Glu Met<br>275 280 285     | 1136 |
| GCA CTG ACT CTG CAA AGC TAC ATC AAG GGC CAG CAG CGA AGG CCC CGG<br>Ala Leu Thr Leu Gln Ser Tyr Ile Lys Gly Gln Gln Arg Arg Pro Arg<br>290 295 300     | 1184 |
| GAT CGG TTT CTG TAT GCG AAG TTG CTA GGC CTG CTG GCT GAG CTC CGG<br>Asp Arg Phe Leu Tyr Ala Lys Leu Leu Gly Leu Leu Ala Glu Leu Arg<br>305 310 315 320 | 1232 |
| AGC ATT AAT GAG GCC TAC GGG TAC CAA ATC CAG CAC ATC CAG GGC CTG<br>Ser Ile Asn Glu Ala Tyr Gly Tyr Gln Ile Gln His Ile Gln Gly Leu<br>325 330 335     | 1280 |
| TCT GCC ATG ATG CCG CTG CTC CAG GAG ATC TGC AGC TGA GGCCATGCTC<br>Ser Ala Met Met Pro Leu Leu Gln Glu Ile Cys Ser<br>340 345                          | 1329 |
| ACTTCCTTCC CCAGCTCACC TGGAACACCC TGGATACACT GGAGTGGGAA AATGCTGGGA                                                                                     | 1389 |
| CCAAAGATTG GGCCGGGTTC AAAGGGAGCC CAGTGGTTGC AATGAAAGAC TAAAGCAAAA                                                                                     | 1449 |
| C                                                                                                                                                     | 1450 |

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

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

TGYGAGGGNT GYAAGGSNTT YTTYMG

26

- 33 -

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

AAAGGTAAGA TCAGGGACGT GACCGCAG

28

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

TGGGTGAATG AGGACATTAC TGACCGCTCC G

31

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

GGGTAGGTT CACCGAAAGT TCACTCG

27

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

AAAGGTAAGA TCAGGGACGT GACCTCAG

28

- 34 -

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

CTGGAGGTGA CAGGAGGACA GCAGCCCTGA

30

## (2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGACAGGTTG ACCCTTTTTC TAAGGGCTTA ACCTAGCTCA CCTG

44

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Substantially pure CAR receptor polypeptide as herein defined.
2. The polypeptide of claim 1, comprising an amino  
5 acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO:1).
3. A substantially pure CAR receptor polypeptide as herein defined comprising a CAR receptor DNA binding domain and a CAR receptor gene activation domain.
- 10 4. The polypeptide of claim 3, wherein said DNA binding domain comprises a sequence substantially identical to (a) amino acids 11-75 of Fig. 1 (SEQ ID NO:1); or (b) amino acids 76-348 of Fig. 1 (SEQ ID NO:1); or a DNA binding fragment thereof.
- 15 5. A substantially pure polypeptide comprising a CAR receptor heterodimerization domain, said polypeptide having the ability to bind to the amino acid sequence of SEQ ID NO. 5 in the absence of retinoic acid.
6. The polypeptide of claim 1, comprising an amino  
20 acid sequence identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO:1).
7. The polypeptide of claim 3, wherein said DNA binding domain comprises a sequence identical to amino acids 11-75 of Fig 1 (SEQ ID NO:1).
- 25 8. The polypeptide of claim 3, wherein said gene activation domain comprises a sequence identical to amino acids 76-348 of Fig. 1 (SEQ ID NO:1).
9. Substantially pure DNA which encodes a polypeptide according to any one of claims 1 to 8.
- 30 10. A vector comprising the DNA of claim 9, said vector being capable of directing expression of the protein encoded by said DNA in a vector-containing cell.
11. A cell which contains the DNA of claim 9.
12. A method of producing a recombinant CAR receptor  
35 polypeptide as herein defined or a fragment or analog thereof comprising:



(a) providing a cell transformed with DNA encoding a CAR receptor as herein defined or a deferred fragment or analog thereof positioned for expression in said cell;

5 (b) culturing said transformed cell under conditions for expressing said DNA; and

(c) isolating said recombinant CAR receptor polypeptide.

13. A substantially pure antibody which specifically  
10 binds a polypeptide of claims 1 to 8.

14. A composition comprising a polypeptide according to claims 1 to 8 formulated in a physiologically-acceptable carrier.

15. Use of a polypeptide according to any one of  
15 claims 1 to 8 in the manufacture of a medicament for the treatment of Graves' disease in a mammal, said polypeptide being formulated in a dosage effective to decrease thyroid hormone receptor function.

16. Use of a polypeptide according to any of claims 1  
20 to 8 in the manufacture of a medicament for the treatment of cancer in a mammal, said polypeptide being formulated in a dosage effective to increase retinoic acid receptor expression.

17. A method of identifying a CAR ligand, comprising

25 (a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide as herein defined;

(b) introducing into a host cell which is functionally deficient for CAR receptor as herein defined

30 (i) said nucleic acid which encodes said CAR receptor polypeptide and

(ii) a reporter gene operably linked to a CAR receptor polypeptide binding site;

(c) measuring induction of said reporter gene in said transfected host cell;

35 (d) contacting said transfected host cell with a candidate ligand; and



(e) measuring induction of said reporter gene in the presence of said candidate ligand, an increase or decrease in said induction as compared to the induction in (c) being indicative of the presence of a CAR ligand.

5 18. A method of identifying a CAR ligand, comprising

(a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide as herein defined;

(b) introducing said nucleic acid into a host cell such that said recombinant CAR receptor polypeptide  
10 is expressed;

(c) isolating said recombinant polypeptide;

(d) immobilizing said recombinant polypeptide on a solid substrate;

(e) contacting said immobilized recombinant  
15 polypeptide with a candidate ligand under conditions which allow formation of an affinity complex between said immobilized recombinant CAR receptor polypeptide and said candidate ligand; and

(f) detecting complex formation as an  
20 indication of the presence of a CAR ligand.

19. A method of identifying a CAR receptor DNA binding site, comprising

(a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide as herein defined;

25 (b) introducing into a host cell which is functionally deficient for CAR receptor

(i) said nucleic acid which encodes said CAR receptor polypeptide and

(ii) a reporter gene which is operably linked to  
30 a candidate CAR receptor DNA binding site; and

(c) measuring induction of said reporter gene in said transfected host cell, induction being indicative of the presence of an operably linked CAR receptor DNA binding site.

35 20. A method of identifying a CAR receptor DNA binding site, comprising



(a) providing a nucleic acid sequence which encodes a CAR receptor polypeptide is expressed as herein defined;

(b) introducing said nucleic acid into a host cell so that said recombinant CAR receptor polypeptide is expressed;

(c) isolating said recombinant protein;

(d) contacting said recombinant protein with a candidate DNA binding site under conditions which allow formation of an affinity complex between said recombinant CAR receptor polypeptide and said candidate binding site; and

(e) detecting complex formation as an indication of the presence of a CAR receptor DNA binding site.

21. A chimeric receptor comprising the DNA binding domain of a CAR receptor polypeptide, as herein defined, fused to the gene activation domain of a heterologous protein.

DATED this 11<sup>th</sup> day of September 1997

THE GENERAL HOSPITAL CORPORATION

By Their Patent Attorneys:

GRIFFITH HACK

Fellows Institute of Patent Attorneys of Australia



1/1

1 GTGAGCTTGCTCCTTAAGTTACAGGAACCTCTCCTTATAATAGACACTTCA TTTTCCZAGTCCATCCCTCA  
71 TGAAAAATGACTG...ACTGCTGGGCAGCAGGAGGGATGATAATCCTA...TCCAATCACTGGCAACTCCT  
141 GAGATCAGAGGAAAACCAGCAACAGCGTGGGAGTTTGGGGAGAGGCATTCCATACCAGATTCTGTGCGCT  
211 GCAGGTGACATGCTGCCTAAGAGAAGCAGGAGTCTGTGACAGCCACCCCAACAGGTGAAGTCATGGCCAG  
N A S  
281 TAGGGAAGATGAGCTGAGGAACCTGTGTGGTATGTGGGGACCAAGCCACAGGCTACCACCTTTAATGGGCTO  
R E D E L R N C V V C G D Q A T G Y H F N A L  
351 ACTTGTGAGGGCTGCAAGGGTTTCTTCAGGAGAACAGTCAGCAAAAGCATTGGTCCCACTGCCCCCTTTG  
T C E G C K G F F R R T V S K S I G P T C P F A  
421 CTGGAAGCTGTGAAGTCAGCAAGACTCAGAGGCCCACTGCCAGCCTGCAGGTTGCAGAAGTGTCTAGA  
G S C E V S K T Q R R H C P A C R L Q K C L D  
491 TGCTGGCATGAGGAAAGACATGATACTGTGGCAGAAGCCCTGGCATTGGGGGAGCAAGCAGGCCAG  
A G H R K D M I L S A E A L A L R R A K Q A Q  
561 CGGCGGGCACAGCAAAACCTGTGCAACTGAGTAAGGAGCAAGAAGAGCTGATCCGGACACTCCTGGGGG  
R R A Q Q T P V Q L S K E Q E E L I R T L L Q A  
631 CCCACACCGGCCACATGGGCACCATGTTTGAACAGTTTGTGCAGTTTAGGCCCTCAGCTCATCTGTTCA  
H T R H M G T M F E Q F V Q F R P F A H L F I  
701 CCATCACCAGCCCTTGCCGACCTGGCCCTGTGCTGCTCTGGTCACACACTTGGCAGACATCAACACT  
H H Q P L P T L A P V L P L V T H F A D I N T  
771 TTCATGGTACTGCAAGTCATCAAGTTTACTAAGGACCTGCCCTCTTCCGTTCCCTGCCCATTTGAAGAC  
P M V L Q V I K F T R D L P V F R S L P I E D Q  
841 AGATCTCCCTTCTCAAGGGAGCAGCTGTGGAAATCTGTACATCGTACTCAATACCACCTTTCTGTCTCCA  
I S L L K G A A V E I C H I V L N T T F C L Q  
911 AACACAAAACCTCCTCTGGGGCTCTTCCCTACACAATTGAAGATGGAGCCCTGTGGGGTTCCAGGTA  
T Q N F L C G P L R Y T I E D G A R V G F Q V  
981 GAGTTTTTGGAGTTGCTCTTTCACTTCCATGGAACACTACGAAAACCTGCAGCTCCAAGAGCCTGAGDATG  
E F L E L L F H F H G T L R K L Q L Q E P E Y W  
1051 TGCTCTTGGCTGCCATGGCCCTCTTCTCTCTGACCGACTGGAGTTACCCAGAGAGATGAGATTGATCA  
L L A A H A L F S P D R P G V T Q R D E I D Q  
1121 GCTGCAAGAGGAGATGGCACTGACTCTGCAAGCTACATCAAGGGCCAGCAGCGAAGGCCCGGGATCGG  
L Q E E H A L T L Q S Y I K G Q Q R R P R D R  
1191 TTTCTGTATGCGAAGTTGCTAGGCTGCTGGCTGAGCTCCGGAGCATTAATGAAGCCCTAAGGGTACCAAA  
F L Y A K L L G L L A E L R S I N E A Y G Y Q I  
1261 TCCAGCACATCCAGGGCTGTCTGCCATGATGCCGTGCTCCAGGAGATCTGCAGCTGAGGCCATGCTCA  
Q H I Q G L S A M H P L L Q E I C S \*  
1331 CTTCTTCCCCAGCTCACTGGAACACCTGGATACACTGGAGTGGGAAAATGCTGGGACCAAGATTGG  
1401 GCGGGGTTCAAAGGGAGCCCACTGGTTGCAATGAAGACTAAAGCAAAAC 1450

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FIG. 1

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01559**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : 530/350, 387.1; 514/2; 536/23.5; 435/340.1, 240.2, 69.1, 6, 7.6

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.1; 514/2; 536/23.5; 435/340.1, 240.2, 69.1, 6, 7.6

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

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

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category*       | Citation of document, with indication, where appropriate, of the relevant passages                                                                      | Relevant to claim No. |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y               | Cell, Volume 51, issued 24 December 1987, V. Kumar et al., "Functional Domains of the Human Estrogen Receptor", pages 941-950, especially the abstract. | 1                     |
| <u>Y,P</u><br>A | US, A, 5,183,736 (Pfahl et al.) 02 February 1993, abstract and paragraph bridging cols. 1-2.                                                            | <u>1</u><br>2-18      |
| A               | US, A, 5,071,773 (Evans et al.) 10 December 1991.                                                                                                       | 14, 16                |
| A               | US, A, 5,077,211 (Yarosh) 31 December 1991.                                                                                                             | 15, 17                |
| A               | US, A, 4,981,784 (Evans et al.) 01 January 1991.                                                                                                        | 18                    |

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

|                                                                                                                                                                         |     |                                                                                                                                                                                                                                              |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                                | *T  | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| *A* document defining the general state of the art which is not considered to be part of particular relevance                                                           | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| *E* earlier document published on or after the international filing date                                                                                                | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, each combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* | document member of the same patent family                                                                                                                                                                                                    |
| *O* document referring to an oral disclosure, use, exhibition or other means                                                                                            |     |                                                                                                                                                                                                                                              |
| *P* document published prior to the international filing date but later than the priority date claimed                                                                  |     |                                                                                                                                                                                                                                              |

|                                                                                                                       |                                                                          |
|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Date of the actual completion of the international search<br>29 April 1993                                            | Date of mailing of the international search report<br><b>06 MAY 1993</b> |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>DAVID L. FITZGERALD                                |
| Facsimile No. NOT APPLICABLE                                                                                          | Telephone No. (703) 308-0196                                             |

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01559

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                            | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | Oncogene, Volume 6, Number 10, issued October 1991, J. F. Gebert et al., "High frequency of retinoic acid receptor beta abnormalities in human lung cancer", pages 1859-1868. | 13                    |
| A         | Science, Volume 240, issued 13 May 1988, R. M. Evans, "The Steroid and Thyroid Hormone Receptor Superfamily", pages 889-895.                                                  | 1-18                  |
| A         | FASEB Journal, Volume 5, issued December 1991, P. J. Fuller, "The steroid receptor superfamily: mechanisms of diversity", pages 3092-3099.                                    | 1-18                  |

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01559

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07K 13/00; A61K 37/00; C12N 15/12, 15/63, 15/65; C12P 21/08; C12Q 1/68, 1/25

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

SEQUENCE DATABASES: GenBank, EMBL, GeneSeq, PIR, SwissProt

KEYWORD DATABASES: US PTO-APS; Medline

SEARCH TERMS: steroid, retino?, receptor; constitutive?()activat?; Graves, cancer