



(72) ADAMS, John S., US

(71) CEDARS-SINAI MEDICAL CENTER, US

(51) Int.Cl.⁶ C07K 14/47, G01N 33/53, C07K 1/22, C07K 16/18, A61K 38/17

(30) 1996/02/12 (60/011,482) US

(54) **PROETINES DE LIAISON D'ELEMENTS DE REPONSE AUX
OESTROGENES ET NUCLEOTIDES LES CODANT**

(54) **ESTROGEN RESPONSE ELEMENT BINDING PROTEINS AND
NUCLEOTIDES ENCODING THEREFOR**

(57) L'invention a trait à la découverte et à la purification de nouvelles protéines de liaison d'éléments de réponse aux oestrogènes. Les protéines de liaison d'éléments de réponse aux oestrogènes sont distinctes du récepteur d'oestrogènes et d'autres récepteurs intracellulaires, par exemple le récepteur d'oestrogènes. Les protéines de liaison d'éléments de réponse aux oestrogènes peuvent perturber l'activité biologique du récepteur d'oestrogènes ainsi que d'autres récepteurs protéiques intracellulaires apparentés. Un aspect de l'invention a trait à des protéines de liaison purifiées d'éléments de réponse aux oestrogènes. Un autre aspect de l'invention a trait à des anticorps capables de se lier aux protéines de liaison d'éléments de réponse aux oestrogènes de l'invention. Un autre aspect de l'invention concerne des dosages permettant la détection ou le criblage de composés thérapeutiques perturbant l'interaction entre une protéine de liaison d'éléments de réponse aux oestrogènes et des éléments de réponse aux oestrogènes.

(57) The invention relates to the discovery and purification of novel estrogen response element binding proteins. Estrogen response element binding proteins are distinct from the estrogen receptor and other intracellular receptors, e.g. estrogen receptor. Estrogen response element binding proteins can interfere with the biological activity of the estrogen receptor and other related intracellular receptor proteins. One aspect of the invention is to provide purified estrogen response element binding proteins. Another aspect of the invention is to provide antibodies capable of binding to the estrogen response element binding proteins of the invention. Another aspect of the invention is to provide assays for the detection or screening of therapeutic compounds that interfere with the interaction between estrogen response element binding protein and estrogen response elements.

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/12, C07K 14/46, 14/47, 16/18, A61K 39/395	A1	(11) International Publication Number: WO 97/29184 (43) International Publication Date: 14 August 1997 (14.08.97)
(21) International Application Number: PCT/US97/02208 (22) International Filing Date: 12 February 1997 (12.02.97) (30) Priority Data: 60/011,482 12 February 1996 (12.02.96) US (71) Applicant: CEDARS-SINAI MEDICAL CENTER [US/US]; 8700 Beverly Boulevard, Los Angeles, CA 90048 (US). (72) Inventor: ADAMS, John, S.; 16422 Sloan Drive, Los Angeles, CA 90049 (US). (74) Agents: HALLUIN, Albert, P. et al.; Pennie & Edmonds L.L.P., 1155 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ESTROGEN RESPONSE ELEMENT BINDING PROTEINS AND NUCLEOTIDES ENCODING THEREFOR (57) Abstract The invention relates to the discovery and purification of novel estrogen response element binding proteins. Estrogen response element binding proteins are distinct from the estrogen receptor and other intracellular receptors, e.g. estrogen receptor. Estrogen response element binding proteins can interfere with the biological activity of the estrogen receptor and other related intracellular receptor proteins. One aspect of the invention is to provide purified estrogen response element binding proteins. Another aspect of the invention is to provide antibodies capable of binding to the estrogen response element binding proteins of the invention. Another aspect of the invention is to provide assays for the detection or screening of therapeutic compounds that interfere with the interaction between estrogen response element binding protein and estrogen response elements.		

**ESTROGEN RESPONSE ELEMENT BINDING PROTEINS AND
NUCLEOTIDES ENCODING THEREFOR**

1.0. Field of the Invention

5 The invention is in the field of estrogen signaling.
Portions of the invention described herein were made in the
course of research supported in part by NIH grants AR37399
and DK07682. The Government may have certain rights in this
10 invention.

2.0. Background

15 Estrogens and other related steroids (both natural and
synthetic) that bind to estrogen receptors have profound
physiological effects in humans and other animals. These
effects include decreasing the rate of bone resorption,
altering the circulating levels of clotting factors,
stimulating breast development, and many others. Detailed
descriptions of the physiological effects of estrogens can be
20 found in many standard text books, including Goodman and
Gilman's, The Pharmacological Basis of Therapeutics. Eight
Edition and Goodman, et al. Pergaman Press Elmsford, NY
(1990).

25 Estrogens are known to bind to an intracellular estrogen
receptor (ER). Intracellular estrogen receptors are known to
belong to a superfamily of intracellular receptors that
comprise DNA binding domains. Estrogen receptors bind to the
consensus estrogen response element 5'-AGGTCACAGTGACCT, see
Tsai, et al., Annu. Rev. Biochem 63:451-486 (1994).

30 The invention described herein relates to the discovery
of a novel protein that inhibits the binding of estrogen
receptor to the estrogen response element (ERE). By
inhibiting the binding of the estrogen receptor to estrogen
response element, it is possible to modulate the expression
35 of genes regulated by estrogen via estrogen receptors. For
example, estrogen response element binding protein may be
used to modulate fertility. Additionally, estrogen and

estrogen receptors are known to play a significant role in the etiology of many diseases such as osteoporosis and cancer, e.g. estrogen responsive tumors. It is thus of interest to provide proteins that interact with estrogen response elements and polynucleotides encoding such proteins.

3.0. Summary of the Invention

The invention relates to the discovery and purification of novel estrogen response element binding proteins (ERE-BP) and the isolation of polynucleotide sequences encoding the proteins. Estrogen response element binding proteins are of interest because they may mediate the high levels steroid hormones observed in new world primates. Estrogen response element binding proteins are distinct from the estrogen receptor and other intracellular receptors, e.g. estrogen receptor. Estrogen response element binding proteins can interfere with the biological activity of the estrogen receptor and other related intracellular receptor proteins. Estrogen response element binding proteins of the present invention can bind to a DNA sequence known as the estrogen response element that is conserved (or partially conserved) among the regulatory regions of estrogen regulated genes.

By binding to estrogen response elements, estrogen response element binding proteins prevent estrogen receptor complex from binding the response element. These properties of estrogen response element binding proteins have profound physiological effects. Thus by regulating the intracellular levels of the subject estrogen response element binding proteins, desirable physiological may be obtained.

One aspect of the invention is to provide substantially purified estrogen response element binding proteins. The purified proteins may be obtained from either recombinant cells or naturally occurring cells. The purified estrogen response element binding proteins of the invention may be mammalian in origin. Estrogen response element binding proteins derived from primates, including human and *Callithrix jacchus* (common marmoset), are examples of the

various estrogen response element binding proteins specifically provided for in the present application.

The invention also provides allelic variants and biologically active derivatives of naturally occurring
5 estrogen response element binding proteins.

Another aspect of the invention is to provide polynucleotides encoding the estrogen response element binding proteins of the invention and to provide polynucleotides complementary to polynucleotide coding
10 strand. The polynucleotides of the invention may be used to provide for the recombinant expression of estrogen response element binding proteins. The polynucleotides of the invention may also be used for genetic therapy purposes so as to treat diseases related to estrogen receptors and ligands
15 that bind to estrogen receptors.

The present invention also provides polynucleotides for use as hybridization probes and amplification primers for the detection of naturally occurring polynucleotides encoding estrogen response element binding proteins.

20 Another aspect of the invention is to provide antibodies capable of binding to the estrogen response element binding proteins of the invention. The antibodies may be polyclonal or monoclonal. The invention also provides methods of using the subject antibodies to detect and measure expression of
25 estrogen response element binding protein either *in vitro* or *in vivo*.

Another aspect of the invention is to provide assays for the detection or screening of therapeutic compounds that interfere with the interaction between estrogen response
30 element binding protein and estrogen response elements. The assays of the invention comprise the step of measuring the effect of a compound of interest on binding between estrogen response element binding protein and an estrogen response element. Binding may be measured in a variety of ways,
35 including the use of labeled estrogen response element binding protein or labeled DNA sequences comprising an estrogen response binding element.

4.0. Description of the Specific Embodiments

The invention relates to the discovery and purification of novel estrogen response element binding proteins. Estrogen response element binding proteins are of interest because, *inter alia*, they modulate the activity of estrogen receptors. Estrogen response element binding proteins are distinct from the estrogen receptor. Estrogen response element binding proteins can interfere with the activity of the estrogen receptor by binding to the same DNA sequence, i.e., the estrogen receptor binding element. Thus by regulating the intracellular levels of the subject estrogen response element binding proteins, physiological effects of interest may be obtained. Such effects may be used to modulate fertility, or to treat a variety of diseases involving the signaling at intracellular receptors including osteoporosis, glucocorticoid mediated disorders, hypercalcemia, granuloma forming diseases, and estrogen responsive tumors or proliferative disorders.

The estrogen response element binding proteins of the invention have the biological activity of specifically binding to estrogen response element DNA sequences, including the estrogen response element consensus sequence and estrogen response element from one or more genes.

The estrogen response element binding proteins of the invention may be isolated from a variety of mammalian animal species. Preferred mammalian species for isolation are primates, humans and new world primates being particularly preferred. Although humans and old world primates do not produce large enough quantities of estrogen response element binding protein to manifest the elevated steroid hormone phenomenon seen in new world primates, humans and old world primates (as well as other mammals) are believed to produce estrogen response element binding proteins. The invention also contemplates allelic variants of estrogen response element binding protein. Estrogen response element binding proteins may be prepared from a variety of mammalian tissues; however, leukocytes and cell lines established from blood

leukocytes are preferred non-recombinant sources of estrogen response element binding proteins.

Preferably, estrogen response element binding proteins are obtained from recombinant host cells genetically engineered to express significant quantities of estrogen response element binding proteins. Estrogen response element binding proteins may be isolated from non-recombinant cells in a variety of ways well known to a person of ordinary skill in the art. One example of such an isolation method is provided below in the examples section. Methods for purifying recombinant proteins from genetically engineered host cells vary with the host cell type and are well known to persons of ordinary skill in the art.

Experiments with estrogen response element binding protein suggest that the protein isolated from B95-8 cell, (see examples section) has a relative molecular weight of about 44-45 kDa (kilodaltons), as determined by SDS-PAGE.

The term "estrogen response element binding protein" as used herein refers not only to proteins having the amino acid residue sequence of naturally occurring estrogen response element binding proteins but also refers to functional derivatives and variants of naturally occurring estrogen response element binding protein.

A "functional derivative" of a native polypeptide is a compound having a qualitative biological activity in common with the native polypeptide. Thus, a functional derivative of a native estrogen response element binding protein is a compound that has a qualitative biological activity in common with a native estrogen response element binding protein, e.g., binding to the ERE and other cognate ligands. More preferably, the functional derivative of ERE-BP is distinct and largely unrelated to ER as evidenced by the fact that the functional derivative will specifically bind the ERE, and, like ERE-BP, will preferably not specifically binding 17 β -estradiol as measured by a electromobility shift assays (EMSA), and will not be recognized by ER-specific antisera.

"Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including humans), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a respective native polypeptide. "Fragments" comprise regions within the sequence of a mature native polypeptide. The term "derivative" is used to define amino acid sequence and glycosylated variants, and covalent modifications of a native polypeptide, whereas the term "variant" refers to amino acid sequence and glycosylated variants within this definition.

Preferably, the functional derivatives are polypeptides which have at least about 65% amino acid sequence identity, more preferably about 75% amino acid sequence identity, even more preferably at least 85% amino acid sequence identity, most preferably at least about 95% amino acid sequence identity with the sequence of a corresponding native polypeptide. Most preferably, the functional derivatives of a native estrogen response element binding protein retain or mimic the region or regions within the native polypeptide sequence that directly participate in ligand binding. An additional feature of such functional derivatives is that they are not substantially bound or hindered by anti-ER antibodies as demonstrated by EMSA. Functional derivatives of ERE-BP also include chemically modified or derivatized molecules derived from ERE-BP.

The phrase "functional derivative" further and specifically includes peptides and small organic molecules having a qualitative biological activity in common with a native estrogen response element binding protein.

"Identity" or "homology" with respect to a native polypeptide and its functional derivative is defined herein as the percentage of amino acid residues in the candidate sequence that are identical to the corresponding residues of a native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative

substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art.

5 Once a gene encoding an ERE-BP activity, or functional derivative thereof, has been identified and cloned, amino acid sequence variants of native estrogen response element binding proteins and estrogen response element binding protein fragments are prepared by methods known in the art by
10 introducing appropriate nucleotide changes into a native or variant estrogen response element binding protein encoding DNA, or by *in vitro* synthesis of the desired polypeptide. There are two principal variables in the construction of amino acid sequence variants: the location of the mutation
15 site and the nature of the mutation. With the exception of naturally-occurring alleles, which do not require the manipulation of the DNA sequence encoding the estrogen response element binding protein, the amino acid sequence variants of estrogen response element protein are preferably
20 constructed by mutating the ERE-BP encoding DNA to generate corresponding ERE-BP amino acid sequence variants that do not occur in nature.

Such mutants may be engineered, for example, as frame-shift mutations that result in an altered reading frame and
25 early termination of translation to produce a truncated ERE-BP molecule. Similarly, in-frame deletions may be made in the ERE-BP gene that effectively result in the removal of discrete portions of ERE-BP. Such amino acid sequence deletions generally range from about 1 to 30 residues, more
30 preferably about 1 to 10 residues, and are typically, but not necessarily, contiguous. Deletions are generally introduced in regions that are not directly involved in ligand binding.

Alternatively or in addition, amino acid alterations can be made at sites that differ in estrogen response element
35 binding proteins from various species, or in highly conserved regions, depending on the goal to be achieved.

Sites at such locations will typically be modified in series, e.g. by (1) substituting first with conservative choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue or
5 residues, or (3) inserting residues of the same or different class adjacent to the located site, or combinations of options 1-3.

One helpful technique is called "alanine scanning" Cunningham and Wells, Science 244, 1081-1085 (1989). Here, a
10 residue or group of target residues is identified and substituted by alanine or polyalanine. Those domains demonstrating functional sensitivity to the alanine substitutions are then refined by introducing further or other substituents at or for the sites of alanine
15 substitution.

After identifying the desired mutation(s), the gene encoding an estrogen response element binding protein variant can, for example, be obtained by chemical synthesis..

More preferably, DNA encoding an estrogen response
20 element binding protein amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes are earlier prepared variant or a nonvariant version of the estrogen response element binding protein. Site-directed (site-specific) mutagenesis allows the production of estrogen
25 response element binding protein variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable
30 duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 50 nucleotides in length is preferred, with at least about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are
35 well known in the art, as exemplified by publications such as, Edelman et al., DNA 2:183 (1983). As will be appreciated, the site-specific mutagenesis technique

typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. This and other phage vectors are commercially available and their use is well known to those skilled in the art. A versatile and efficient procedure for the construction of oligodeoxyribonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller, M.J. and Smith, M., Nucleic Acids Res. 10, 6487-6500, 1982. Also, plasmid vectors that contain a single-stranded phage origin of replication, Veira et al., Meth. Enzymol. 153:3 (1987) may be employed to obtain single-stranded DNA. Alternatively, nucleotide substitutions are introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis may be performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea et al., Proc. Natl. Acad. Sci. USA 75, 5765 (1978). This primer is then annealed with the single-stranded protein sequence-containing vector, and subjected to DNA-polymerizing enzymes such as, *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells such as HB101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region may be removed and placed in an appropriate expression vector for protein production.

The PCR technique may also be used in creating amino acid sequence variants of an estrogen response element binding protein. When small amounts of template DNA are used

as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primes can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

Further details of the foregoing and similar mutagenesis techniques are found in general textbooks, such as, for example, Sambrook et al., Molecular Cloning: H Laboratory Manual 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor (1989), and Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley and Sons (1995).

Naturally-occurring amino acids may be divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophobic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another (see generally

Orcutt, B.C. and Dayhoff, M.O., Scoring Matrices, PIN Report MAT-0285, February 1985). Variants obtained by non-conservative substitutions are expected to result in significant changes in the biological properties/function of the obtained variant, and may result in estrogen response element binding protein variants which block estrogen response element binding protein biological activities, i.e., binding to estrogen response elements. Amino acid positions that are conserved among various species are generally substituted in a relatively conservative manner if the goal is to retain biological function.

Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e. insertions within the estrogen response element binding protein amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5 residues, more preferably 1 to 3 residues. Examples of terminal insertions include the estrogen response element binding proteins with an N-terminal methionyl residue, an artifact of direct expression in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the estrogen response element binding protein to facilitate the secretion of the mature estrogen response element binding protein from recombinant host cells. Such signal sequences will generally be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertional variants of the native estrogen response element binding protein molecules include the fusion of the N- or C-terminus of an estrogen response element binding protein to immunogenic polypeptides, e.g. bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, and C-terminal fusions with proteins having

a long half-life such as immunoglobulin regions (preferably immunoglobulin constant regions), albumin, or ferritin, as described in PCT published application WO 89/02922.

Since it is often difficult to predict in advance the characteristics of a variant estrogen response element binding protein, it will be appreciated that screening will be needed to select the optimum variant. For this purpose biochemical screening assays, such as those described hereinbelow, will be readily available.

Several steroid hormone responsive diseases may be treated through either *in vivo* or *in vitro* genetic therapy. Protocols for genetic therapy through the use of viral vectors can be found, among other places, in Viral Vector Gene Therapy and Neuroscience Applications, Kaplit and Lowry, Academic Press, San Diego (1995). The genetic therapy methods of the invention comprise the step of introducing a vector for the expression of estrogen response element binding protein (or inhibitory anti-sense RNA) into a patient cell. The patient cell may be either in the patient, i.e., *in vivo* genetic therapy, or external to the patient and subsequently reintroduced into the patient, i.e., *in vitro* genetic therapy. Diseases that may be treated by the subject genetic therapy methods include osteoporosis, vitamin D toxicity, glucocorticoid hormone overproduction, sex steroid hormone over expression and under expression, hypercalcemia (attributable to vitamin D over expression), granuloma forming diseases and the like.

The subject invention provides methods for the treatment of a variety of diseases characterized by undesirably high levels of estrogen or other steroids that can bind to estrogen response element binding proteins of the invention.

One of ordinary skill will appreciate that, from a medical practitioner's or patient's perspective, virtually any alleviation or prevention of an undesirable symptom (e.g., symptoms related to disease, sensitivity to environmental or factors, normal aging, and the like) would be desirable. Thus, for the purposes of this Application,

the terms "treatment", "therapeutic use", or "medicinal use" used herein shall refer to any and all uses of the claimed compositions which remedy a disease state or symptoms, or otherwise prevent, hinder, retard, or reverse the progression of disease or other undesirable symptoms in any way whatsoever.

When used in the therapeutic treatment of disease, an appropriate dosage of ERE-BP, or a functional derivative thereof, may be determined by any of several well established methodologies. For instance, animal studies are commonly used to determine the maximal tolerable dose, or MTD, of bioactive agent per kilogram weight. In general, at least one of the animal species tested is mammalian. Those skilled in the art regularly extrapolate doses for efficacy and avoiding toxicity to other species, including human. Before human studies of efficacy are undertaken, Phase I clinical studies in normal subjects help establish safe doses.

Where diagnostic, therapeutic or medicinal use of ERE-BPs, or derivatives thereof, is contemplated, the ERE-BP may be prepared and maintained under sterile conditions and thus avoid microbial contamination. Compositions comprising ERE-BPs may also be sterile filtered prior to use. In addition to the above methods of sterile preparation and filter sterilization, antimicrobial agents may also be added. Antimicrobial agents which may be used, generally in amounts of up to about 3% w/v, preferably from about 0.5 to 2.5%, of the total formulation, include, but are not limited to, methylparaben, ethylparaben, propylparaben, butylparaben, phenol, dehydroacetic acid, phenylethyl alcohol, sodium benzoate, sorbic acid, thymol, thimerosal, sodium dehydroacetate, benzyl alcohol, cresol, p-chloro-m-cresol, chlorobutanol, phenylmercuric acetate, phenylmercuric borate, phenylmercuric nitrate and benzylalkonium chloride. Preferably, anti-microbial additives will either enhance the biochemical properties of ERE-BP, or will be inert with respect ERE-BP activity. To the extent that a given anti-microbial agent may prove deleterious to ERE-BP activity,

another agent may be substituted which effects ERE-BP function to a lesser extent.

Compositions comprising ERE-BPs as active components may be introduced *in vivo* by any of a number of established
5 methods. For instance, the agent may be administered by inhalation; by subcutaneous (sub-q); intravenous (I.V.), intraperitoneal (I.P.), or intramuscular (I.M.) injection; rectally, as a topically applied agent (transdermal patch, ointments, creams, salves, eye drops, and the like), or
10 directly injected into tissue such as tumors or other organs, or in or around the viscera.

Additionally, ERE-BPs, or functional derivatives thereof, are conjugated to ligands that facilitate the delivery of ERE-BPs to specifically targeted cells or
15 tissues. Examples of such targeting ligands include, but are not limited to, glycoproteins, polysaccharides, lectins, cell receptors or surface markers (or fragments thereof) antibodies (or fragments thereof), aptameric oligonucleotides, and the like.

20 Another aspect of the invention is to provide assays useful for determining if a compound of interest can bind to estrogen response element binding proteins so as to interfere with the binding of an estrogen response element to estrogen response element receptor proteins. The assay comprises the
25 steps of measuring the binding of a compound of interest to an estrogen response element binding protein. Either the intracellular binding protein or the compound of interest to be assayed may be labeled with a detectable label, e.g., a radioactive or fluorescent label, so as to provide for the
30 detection of complex formation between the compound of interest and the estrogen response element binding protein. In another embodiment of the subject assays, the assays involve measuring the interference, i.e., competitive binding, of a compound of interest with the binding
35 interaction between an estrogen response element binding protein and an estrogen response element. For example, the effect of increasing quantities of a compound of interest on

the formation of complexes between radioactivity labeled ERE and an estrogen response element binding protein may be measured by quantifying the formation of labeled ligand-estrogen response element binding protein complex formation.

5 Additional methods of measuring ligand binding to estrogen response element binding proteins can be found in the example section below.

Polyclonal antibodies to estrogen response element binding proteins generally are raised in animals by multiple

10 subcutaneous (sc) or intraperitoneal (ip) injections of an estrogen response element binding protein and an adjuvant. Evolutionarily conserved proteins often share a high degree of interspecies homology. This high level of homology may render a given protein substantially nonimmunogenic when used

15 during attempts to generate polyclonal antisera against a given protein. In such situations, it may be useful to conjugate the protein (e.g., estrogen response element binding protein) or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species

20 to be immunized, e.g. keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine

25 residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}_1\text{N}=\text{C}=\text{NR}$, where R and R_1 are different alkyl groups. Alternatively, denatured protein may be used as an immunizing agent.

Animals are immunized against the immunogenic conjugates

30 or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's

35 complete adjuvant by subcutaneous injection at multiple sites. 7 to 14 days later the animals are bled and the serum is assayed for anti-estrogen response element binding

proteins antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with a conjugate of the same estrogen response element binding protein that has been conjugated to a different protein and/or through a
5 different cross-linking reagent. Conjugates can also be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies are obtained from a population of
10 substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete
15 antibodies. For example, the anti-estrogen response element binding protein monoclonal antibodies of the invention may be made using the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods [Cabilly, et al, U.S. Pat. No.
20 4,816,567].

In the hybridoma method, a mouse or other appropriate host animal, such a hamster is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the
25 protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103
30 (academic Press, 1986)].

The anti-estrogen response element binding protein specific antibodies of the invention have a number of uses. The antibodies may be used to purify estrogen response element binding proteins from either recombinant or non-
35 recombinant cells. The subject antibodies may be used to detect and/or quantify the presence of estrogen response element binding proteins in tissue samples, e.g., from blood,

skin, and the like. Quantitative measurements of estrogen response element binding proteins may be used diagnostically for those diseases and physiological or genetic conditions that have been correlated with particular levels of estrogen response element binding protein expression levels. The invention having been described above, may be better understood by referring to the following examples. The following examples are offered for the purpose of illustrating the invention and should not be interpreted as a limitation of the invention.

5.0. EXAMPLES

5.1. Cell Culture

The B-lymphoblastoid cell line B95-8 and MLA-144 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Both cell lines were maintained in RPMI-1640 medium (Irvine scientific Irvine, CA) routinely supplemented with 10% fetal calf serum (FCSI Gemini Bioproducts, Calabasas, CA), 100 ug/ml streptomycin, 2 mM L-glutamine (both from GIBCO-BRL, Grand Island, NY) and in atmosphere of 95% air, 5% CO₂. In some experiments, confluent cultures were preincubated overnight in medium containing 100 nM 17 β -estradiol, 25-hydroxyvitamin D₃ (25-OHD₃) and 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) prior to harvest and extract preparation.

5.2. Preparation of Cellular Extracts

Postnuclear extracts of each cell line were prepared as previously described in Gacad et al. J. Clin. Invest. 87:996-1001 (1991). Briefly, confluent B95-8 cells were harvested by trypsinization and MLA-144 cells by centrifugation, respectively. Harvested cells were washed twice in ice-cold phosphate-buffered saline (PBS) and twice with ETD buffer (1 mM EDTA, 10 mM Tris-HCl, 5 mM dithiothreitol [DTT]; pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells pellets were then resuspended in ETD buffer and homogenized on ice in five 10-second bursts. Nuclei, with

associated nuclear steroid receptor proteins, were pelleted at 4000xg for 30 minutes at 4°C. The supernatant of this spin was filtered through a Microcon-30 filter (molecular weight cut off 30 kDa; Amicon, Beverly, MA) for the purposes of desalting and concentrating prior to being aliquoted and stored at -70°C.

Nuclear extracts were prepared as described by Zervitz and Akusjarvi Gene Anal. Tech 6:101-109 (1989). Cultured B95-8 and MLA-144 cells were harvested as previously described. Cells were washed twice in ice-cold PBS, washed once in buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM spermine), resuspended in buffer A and allowed to swell at room temperature for 5 minutes. Lysis of the cell membrane was achieved by addition of lysolecithin to a concentration of 400 mg/ml and gentle repetitive inversion of the cells for 90 seconds. Cells membrane lysis was terminated by addition of two volumes of ice-cold buffer B (buffer A containing 3% BSA). All subsequent steps were performed at 4°C. Nuclei were initially pelleted at 1000xg for 30 seconds and then repelleted at 25,000xg for 60 seconds. The resultant nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 0.6 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% [v/v] glycerol) and the nuclear membrane disrupted by repetitive passage through a 23-gauge needle. The homogenate was gently stirred on ice for 30 minutes and then centrifuged for 30 minutes at 25,000xg. The supernatant, designated nuclear extract, was dialyzed against buffer D (20 mM HEPES [pH 7.9], 0.1 M KCl, 0.2 M EDTA, 0.5 mM DTT, 0.5 mM DTT, 0.5 mM PMSF in 20% glycerol) for 30 hours with three exchanges. The nuclear extract was cleared by centrifugation at 25,000xg for 20 minutes, aliquoted, and stored at 70°C. The protein concentration of nuclear and postnuclear extracts were determined by the method of Bradford Anal. Biochem 72:248-254 (1976).

35

5.3. Electromobility Shift Assay (EMSA)

Synthetic oligonucleotides representing various forms of consensus steroid response elements were either prepared at the Molecular Biology Core Facility of the Cedars-Sinai Research Institute or purchased from Promega (Madison, WI). Sequences for the various oligonucleotides were as follows: consensus estrogen response element (ERE) 5'-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3'; ERE half-site 5'-CTAGAAAGTCAGGTCACAGGATCAAT-3'; AGGTCA half-site direct repeat (DR3) 5'-CTAGTGCTCGGGTAGAGGTCAAGGTCACTCGACTCGT-3'; osteopontin vitamin D response element (VDRE) 5'-CTAGTGGGGCTCGGGTAGGGGTTCA-CGAGGTTCACTCGACTCGT-3'; and the irrelevant CTF/NF1 probe 5'-CCTTTGGCATGCTGCCAATATG-3'. Overexpressed wild-type human estrogen receptor (ER) was recovered in extracts of *S. Cerevisiae* as described in Pham, et al., Mol. Endocrinol., 6:1043-1050 (1992). Anti-estrogen receptor antibody was purchased from Santa Cruz BioTech, Santa Cruz, CA. Anti-COUP-TF antibody was a generous gift from M.J. and S.Y. Tsai (Baylor College of Medicine, Houston, TX). Single-strand oligonucleotides were annealed with their complementary sequences and radiolabeled with ³²P-ATP (DuPont-New England Nuclear, Boston, MA) by T4 Kinase (GIBCO-BRL, Grand Island, NY) to a specific activity of 10⁸ cpm/μg DNA. Postnuclear or nuclear extracts (10 μg protein), preincubated (or not) for 1 hour at 23°C with 100 nM 17β-estradiol, 25 OHD3 or 1,25-(OH)₂D3, were incubated with 2 μg poly-dI-dC (Boehringer-Mannheim, Indianapolis, IN) and 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl₂ in 10% glycerol on ice for 15 minutes, ³²P-labeled probe was added and the incubation continued at room temperature for 15 minutes. Samples of this reaction were subjected to 6% polyacrylamide gel electrophoreses in 0.5 x TBE buffer at 100V. The gels were dried and exposed to Kodak X-OMAT AR film. Densitometry was performed on an Helena EDC densitometer (C. Helena Laboratories, Beaumont, TX).

5.4. Nuclear and Postnuclear Extracts of NWP Cells Contain a Protein Which Binds to ERE

Cellular extracts were prepared identically from both the representative NWP B95-8 and OWP MLA cell line. Prior to
5 extraction, cells were ruptured and intact nuclei separated from cell cytosol in a "low-salt" buffer in order to separate the ER, associated with the nuclear pellet, Tzu Kerman, et al. Mol. Endocrin 8:21-30 (1994), from the post-nuclear supernatant that is enriched in intracellular vitamin D
10 binding protein under these conditions Gacad et al. Bone Min. Res. 8:27-35 (1993). The high-salt" nuclear extract was adjusted to 0.1 M KCl by dialysis, while the "low-salt" nuclear extract was adjusted to same salt concentration by dilution. The post-nuclear and nuclear extracts were matched
15 for protein concentration and used in EMSA in the presence or absence of added human ER. The specific DNA binding potential of nuclear and post-nuclear extracts of steroid-resistant new world primate cells and steroid-responsive old world primate cells was studied using a panel of
20 synthetically prepared oligonucleotides including the consensus ERE (estrogen response element) (AGGTCAcagTGACCT). The experiments demonstrated that post-nuclear extract of steroid-resistant B95-8 cells contained a protein(s) (labeled ERE-BP (estrogen response element-binding protein) which
25 retarded the mobility of the labeled estrogen response element. The presence of this complex was specific for extracts of cells with the steroid-resistant phenotype; the wild-type OWP extract matched for protein concentration did not retard the ERE in this fashion. Compared to the other
30 major, slower migrating band seen in the gel, binding of this protein was most specific for the ERE or its constituent half site AGGTCA. Inclusion of a 100-fold molar excess of either unlabelled ERE or the hexanucleotide half-site AGGTCA
35 successfully competed away any binding of the ERE-BP to the labeled ERE. Coincubation of probe and extract with a 100-fold molar excess of the AGGTCA DR3 (AGGTCAcagAGGTCA) was less effective than either the ERE or the AGGTCA DR3 in

competing the ERE-BP off of the ERE. Coincubation with excess radioinert VDRE (vitamin D response element) as found in the human osteopontin promoter, see Carlbert *et al.* Nature 361:657-660 (1993) exhibited minimal competitive potential
5 for the ERE. The irrelevant CTF/NF1 oligonucleotide was without effect on the ERE-BP-ERE complex. Serial dilution of the extract prior to incubation with probe diminished (1:10) then abolished (1:50) the appearance of the retarded ERE. The results obtained confirms that this protein, or a similar
10 behaving species, was also recovered in the nuclear extract of steroid-resistant new world primate cells but not in the nuclear extract of the steroid-responsive OWP cell line.

15 **5.5. Sterol/Steroid Ligands Do Not Alter The ERE-BP-ERE Interaction**

Modification of steroid receptor proteins by their cognate ligands is often critical in determining the pattern of dimerization of the receptors as well as the response element binding capacity of the receptors. To test whether
20 the interaction of ERE-BP with the ERE was also modified by pre-exposure to potential ligands, steroid-resistant NWP B95-8 cells were exposed to ligand prior to extraction or by exposure to ERE-BP-containing extracts to potential ligands prior to incubation with probe *in vitro*. For these
25 experiments, cells or extracts of cells were preincubated (as described in Gacad *et al.*, 1991, J. Clin. Invest. 87:996-1001) with ER-saturating concentrations of 17β -estradiol (100 nM) or similar concentrations of 25-OHD3 and 1,25-(OH)₂D3, compounds exhibiting the highest affinity for intracellular
30 vitamin D binding protein (IVD-BP), Gacad *et al.* Endocrinology 131:2581-2587 (1992), prior to interaction with the probe.

Neither pre-exposure of cells prior to harvest nor incubation with post-nuclear extracts and nuclear extracts to
35 these ligands prior to interaction with labeled ERE had any significant effect on the mobility of the ERE-BP-ERE complex. Furthermore, incubation of NWP post-nuclear extract with an

anti-ER antibody, which super shifts the ER-ERE complex, had no effect on the mobility or intensity of the ERE-BP-ERE complex, either before or after co-incubation with 100 nM 17β -estradiol. Identical results were obtained in extracts of cells incubated with 100 nM 25-OHD3 and 1,25-(OH)₂D3 prior to EMSA.

5.6. The NWP ERE-BP Competitively Disrupts the ER-ERE Interaction

The estrogen response element binding protein present in NWP cell extracts is functionally capable of rendering the cell estrogen resistant *in vivo*. Consequently, ERE-BP should: [1] be able to bind to the ERE with comparable (or greater) affinity than the ER and/or be present in much higher concentrations than the ER in the cell; and [2] be able to interrupt the normal interaction of the ER with the ERE. As previously noted, when the activated estrogen receptor (ER) was exposed to the ERE the mobility of the ERE through the gel was slowed considerably, much more than when the ERE-BP-containing new world primate nuclear or post-nuclear extract was incubated with the ERE probe. These data suggested that the molecular mass of the ER-ERE complex was relatively greater than the ERE-BP-ERE complex. When the ER and either nuclear or post-nuclear extract were coincubated with the ERE probe, both the ER-ERE and ERE-BP-ERE complexes were identified in the same lane. Dilution of the extract prior to coincubation with the activated ER and labeled ERE shifted the balance of retarded complexes in favor of the ER-ERE. These data clearly show that competition for ERE binding is dependent upon the relative amount of ERE-BP present in the extracts of new world primate cells displaying the steroid resistant phenotype.

It is not known whether the ratio of estrogen receptor element binding protein (ERE-BP) and human estrogen receptor (ER) concentration has matched on a molar basis in the above experiments even though the approximate concentrations of the yeast estrogen receptor extract and new world primate cell

extracts had been previously determined. However, one can estimate that the nuclear extracts from new world primate cells were relatively enriched in ERE-BP as compared to endogenous estrogen receptor given that the endogenous ER-ERE complex was not detected in nuclear extracts from B95-8 cells known to possess a normal complement of ER.

An undiluted preparation of a human ER-containing yeast extract retarded more ERE than a new world primate cell extract containing ERE-BP when matched for protein concentration. In this experiment increasing concentrations of unlabelled ERE were used in competition with the labeled ERE for binding both the estrogen receptor and ERE-BP. An 80-fold increase in the concentrations of unlabeled ERE was required to compete away most of the ER-ERE complex, while a 20-fold molar excess of unlabeled ERE completely eliminated the presence of the ERE-BP-labeled ERE complex. To get a better idea of the relative efficacy of the ER and ERE-BP in binding the ERE, this experiment was repeated using a 1:15 dilution of the ER-containing yeast extract and the same preparation of the NWP ERE-BP-containing extract over a broader dose range of competitive, unlabeled ERE. Under these conditions the density of the ER-ERE complex was observed to be less than, instead of greater than, the density of ERE-BP-ERE complex. A 25-fold increase in the concentration of unlabeled ERE was required to compete away most of the ER from the ER-ERE complex and most of the ERE-BP from the ERE-BP-labeled ERE complex. Densitometric analyses of the EMSA of the two experiments employing undiluted ER and ER diluted 1:15 revealed the same result, a 7-fold excess of unlabeled ERE was required to elicit half-maximal displacement of either the ER or ERE-BP from the ERE.

Using the ER-ERE interaction in an electromobility shift assay (EMSA) as a screen for the presence of such a factor it was discovered that both nuclear extracts containing endogenous ER and postnuclear extracts devoid of ER contained an ER-unrelated protein(s) that interacted directly with a consensus ERE.

5.7. DNA Sequence Specificity for Binding The NWP ERE-BP

The consensus ERE is characterized by an inverted repeat of the six-base pair half site motif AGGTCA (18). In order to determine whether binding of the NWP ERE-BP was specific for ERE, other receptor response elements containing the AGGTCA half site motif were used to compete away labeled ERE-ERE-BP binding. The list of unlabeled competitive oligonucleotides containing the AGGTCA half site included the consensus ERE, TRE, TRE palindrome (TREpal), RXRE, and COUP-TF1E. Of these only a 100-fold excess of ERE completely eliminated labeled ERE-ERE-BP binding. A synthetic oligonucleotide DR₃2, harboring a direct repeat of the AGGTCA half site separated by the trinucleotide cag found in the consensus ERE, was nearly as efficient as the ERE in competing with the labeled ERE probe, and this competitive potential was not related to the sequence flanking the half sites. These results suggested that the AGGTCA half site as well as the intervening cag trinucleotide, not just the AGGTCA half site motif alone, were important in determining ERE-BP binding to the ERE.

The next question was whether the ERE-BP interacted with AGGTGCACag in the absence of a second half-site. To answer this question, a synthetic oligonucleotide was prepared that lacked the 3' AGGTCA of the ERE but retained the 5'AGGTCA and intervening trinucleotide cag found in the consensus ERE. This oligonucleotide was as effective as the consensus ERE in competing with the labeled probe, indicating the ERE-BP may bind to a single core element as a monomer.

30 5.8. Affinity Purification of the NWP ERE-BP

Because the AGGTCAcag motif was as effective as the consensus ERE in competing for ERE-BP binding, sepharose-linked concatamers of the AGGTCAcag motif were used in affinity purification of ERE-BP from post-nuclear extracts of B95-8 cells; this strategy optimized our opportunity to recover DNA-binding proteins that interacted with the half-site AGGTCA as a monomer and diminished chances of recovering

ER that may have been inadvertently retained in our post-nuclear extracts. Chromatographic separation of proteins was achieved through a stepwise salt gradient. Fractions from the mid portion of the gradient (0.4-0.7 M KCl) were enriched
5 in a protein(s) which interacted with the ERE in EMSA. The specific activity of this ERE binding activity was greatest in fractions containing 0.5 M and 0.6 M salt, being 20-30-fold greater than that observed in any of the flanking
10 fractions and 20,000-fold greater than that of the starting material. SDS-PAGE of these two fractions from DNA affinity chromatography disclosed the presence of two distinct silver-stained proteins in the 55-65 kDa range. The protein(s) in the 0.5 M KCl fraction from affinity chromatography could also be distinguished from endogenous estrogen receptor; the
15 purified ERE-BP did not interact in EMSA with any of the anti-ER antibodies tested and did not specifically bind 17 β -estradiol.

The DNA affinity column was prepared essentially as described by Kadondaga and Tijan (16). Two gel-purified
20 oligodeoxynucleotides (30-mers containing 26 nucleotides of complementary sequence and having four base-pair, cohesive ends; 5'-GATCCTA-GAAAGT-CAGGTCA CAGGATCAAT-3' and 5'-GATCATTGATCCTGTGACCTGACTTTC-TAG-3') were synthesized. 220 μ g of each oligonucleotide was annealed, 5'-phosphorylated with
25 [gamma-³²P] ATP to ascertain coupling efficiency, and ligated. The resultant DNA oligomers were then coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia, Piscataway, NJ). After blocking the excess remaining active groups with ethanolamine, the DNA-coupled resin was placed in a 2 ml
30 poly-prep column (BIO-Rad, Hercules, CA) for chromatography. The column was equilibrated in elution buffer (25 mM Hepes [pH 7.6], 12.5 mM MgCl₂, 1 mM DTT, 20% glycerol, and 0.1% nonidet P-40) containing 0.1 M KCl. The B95-8 cell extract (13-20 mg) prepared as above) was solubilized in the elution
35 buffer containing nonspecific competitor poly dIdC (4 mg/ml) and added to the column by gravity flow (12 ml/hour). Protein was then eluted from the column in a stepwise

gradient fashion by the successive addition of ten 10 mL aliquots of elution buffer containing 0.1-1.0 M KCl. A fraction (2 ml) of each column eluate were concentrated and desalted through a Microcon-30 filter and ERE binding capacity assessed by EMSA. An aliquot of each fraction was used to determine total protein concentration. The constituent proteins in each fraction were resolved on a 10% SDS-PAGE and identified by silver staining.

The above experiments have shed insight into the functional aspects of the identified estrogen response element binding protein(s) (ERE-BP) *in vitro*. The ERE-BP does not appear to be related to the ER. Both the ER-ERE and ERE-BP-ERE complexes may be observed as distinct bands in the same gel with the apparent molecular mass of the ERE-BP-ERE complex being less than that of the ER-ERE complex; data suggest that ERE-BP has a molecular mass in the range of 44-45 kDa. Furthermore, the ER and ERE-BP compete for binding to the ERE.

There is additional evidence that the ERE-BP and ER are distinct entities. Notwithstanding the fact that there is a distinct species difference between human and non-human primates, particularly NWP, ERE-BP is not super shifted by anti-ER antibody. Nor is the ERE-BP complex altered by pre-exposure to 17 β -estradiol, the ligand of choice for the ER. Finally, whereas the preferred mode of interaction of the ER with the ERE is as a ligand-transformed homodimer, Tsai, et al., Annu. Rev. Biochem 63:451-486 (1994). ERE-BP may interact with the ERE as a monomer. This is supported by the fact that the labeled ERE-BP-ERE complex can be effectively competed away by addition of the hexanucleotide half-site AGGTCA alone or by a direct repeat of this half-site.

INCORPORATION BY REFERENCE

All patents, patents applications, and publications cited herein are incorporated reference.

WO 97/29184

PCT/US97/02208

EQUIVALENTS

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-
5 described makes for carrying out the invention which are obvious to those skilled in the field of organic chemistry or related fields are intended to be within the scope of the following claims.

10

15

20

25

30

35

CLAIMS

What is claimed is:

1. A substantially purified estrogen response element binding protein.
- 5 2. The estrogen response element binding protein of claim 1, wherein said protein is of mammalian origin.
3. The estrogen response element binding protein of
10 claim 2, wherein the mammal is a primate.
4. The estrogen response element binding protein of claim 3, wherein the primate is human.
- 15 5. The estrogen response element binding protein of claim 3, wherein the primate is *Callithrix jacchus*.
6. A method of purifying an estrogen response element binding protein, comprising: exposing an estrogen response
20 element binding protein to the nucleotide sequence AGGTCA.
7. An antibody having the property of specifically binding an estrogen response element binding protein according to claim 1.
- 25 8. An antibody according to claim 7, wherein said antibody is a monoclonal antibody.
9. A method for detecting an estrogen response element
30 binding protein, said method comprising the steps of:
 - a) contacting cells or cell extracts with an antibody according to claim 7; and
 - b) detecting the formation of complexes formed between said antibodies and estrogen response element binding protein
35 in said cell or cell extracts.

WO 97/29184

PCT/US97/02208

10. The use of the estrogen response element binding protein of claim 1 to provide a therapeutic benefit to an animal.

5 11. The use of claim 10, wherein said therapeutic benefit is the treatment of cancer.

12. The use of claim 10, wherein said therapeutic benefit is the regulation of fertility.

10

13. The use of claim 10, wherein said animal is a mammal.

14. The use of claim 13, wherein said mammal is a
15 primate.

15. The use of claim 14, wherein said primate is a human.

20 16. The use of claim 15, wherein said therapeutic benefit is the treatment of cancer.

17. The use of claim 15, wherein said therapeutic benefit is the regulation of fertility.

25

30

35