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**Modulators of morphogen expression and methods of identifying the same**

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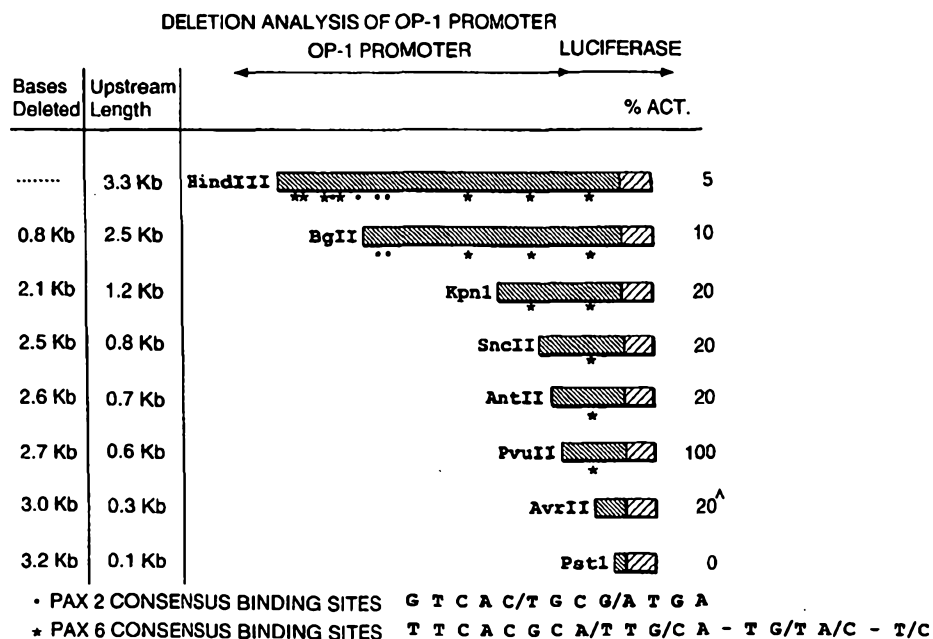
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(54) Title: MODULATORS OF MORPHOGEN EXPRESSION AND METHODS OF IDENTIFYING THE SAME

(57) Abstract

Disclosed are methods and compositions for identifying compounds having an ability to modulate expression of a morphogen, particularly OP-1, OP-1 homologues and closely related proteins, using one or more OP-1-specific, non-coding sequences and a suitable reporter gene. In preferred embodiments, the OP-1-specific non-coding DNA sequence comprises a Pax-responsive OP-1-modulating element.



## Modulators of Morphogen Expression and Methods of Identifying The Same

### Field of the Invention

The invention relates generally to the field of drug screening assays. More particularly, the invention relates to methods and compositions for identifying molecules that modulate  
5 expression of true tissue morphogenic proteins.

### Background of the Invention

A class of proteins recently has been identified, the members of which are true tissue morphogenic proteins. The members of this class of proteins are characterized as competent for inducing the developmental cascade of cellular and molecular events that culminate in the  
10 formation of new organ-specific tissue, including any vascular and connective tissue formation, as required by the naturally occurring tissue. Specifically, the morphogens are competent for inducing all of the following biological functions in a morphogenically permissive environment: (1) stimulating proliferation of progenitor cells; (2) stimulating differentiation of progenitor cells; (3) stimulating the proliferation of differentiated cells and (4) supporting the growth and  
15 maintenance of differentiated cells. For example, the morphogenic proteins can induce the full developmental cascade of bone tissue morphogenesis, including the migration and proliferation of mesenchymal cells, proliferation and differentiation of chondrocytes, cartilage matrix formation and calcification, vascular invasion, osteoblast proliferation, bone formation, bone remodeling, and hematopoietic bone marrow differentiation. These proteins also have been  
20 shown to induce true tissue morphogenesis of non-chondrogenic tissue, including dentin, liver, and nerve tissue.

A particularly useful tissue morphogenic protein is human OP-1 (Osteogenic Protein-1), described in U.S. 5,011,691; US Pat. No. 5,266,683 and Ozkaynak et al. (1990) *EMBO J.* 9: 2085-2093. Species homologues identified to date include, but are not limited to, mouse OP=1  
25 (see US Pat. 5,266,683) and the *Drosophila* homologue 60A, described in Wharton et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9214-9218). Other closely related proteins include OP-2 (Ozkaynak (1992) *J. Biol. Chem.* 267:25220-25227 and US Pat. No. 5,266,683); BMP5, BMP6

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(Celeste et al. (1991) *Proc. Natl. Acad. Sci.* 87:9843-9847) and Vgr-1 (Lyons et al. (1989). These disclosures are incorporated herein by reference.

It previously has been contemplated that these tissue morphogens can be administered to an animal to regenerate lost or damaged tissue. Certain complications, however, presently are encountered during the production, formulation and use *in vivo* of therapeutic macromolecules, such as morphogen proteins. For example, such proteins are typically produced by fermentation or culture of suitable host cells. Any biological product produced from such host cells for use in humans presently must be shown to be essentially free of host cell contaminants, such as secreted or shed proteins, viral particles or degradation products thereof. Providing such assurance can add significantly to the cost and technical difficulty of commercial production of biological macromolecules. Furthermore, appropriate formulations must be developed for conferring commercially reasonable shelf life on the produced macromolecule, without significant loss of biological efficacy. An additional complicating factor arises when circumstances warrant an extended course of therapeutic treatment with the produced and formulated macromolecule: the treated mammal may develop an immunological response to the macromolecule, and any such response may interfere with effectiveness thereof. In extreme circumstances, treatment must be discontinued.

Alternatively, administering a molecule capable of modulating expression of the endogenous tissue morphogen is an effective means for providing morphogen to a site *in vivo*. For example, DNA sequences have been identified in the OP-1 gene promoter that resemble wt-1/Egr-1 consensus sequences, TLC binding sequences, FTZ binding sequences and steroid binding sequences (see WO 95/33831, the disclosure of which is incorporated herein by reference). Thus, molecules to which these regulatory sequences are responsive are likely modulators of the OP-1 gene and can influence its expression.

It is an object of this invention to provide compositions and methods of identifying compounds which can modulate expression of an endogenous tissue morphogen, particularly OP-1 and other members of the larger genus of true tissue morphogens. The compounds thus identified have utility both *in vitro* and *in vivo*. Useful compounds contemplated include at least those that are capable of stimulating transcription and/or translation of the OP-1 gene, as well as

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compounds capable of inhibiting transcription and/or translation of the OP-1 gene, via OP-1 non-coding DNA sequences resembling consensus sequences for Pax homeobox genes, in particular, the Pax 6 or Pax 2 genes.

These and other objects and features of the invention will be apparent from the  
5 description, drawings and claims which follow.

### Summary of the Invention

The invention features compositions and methods for screening candidate compounds for their ability to modulate the effective local or systemic levels of endogenous morphogen, particularly OP-1, in an organism. In one aspect, the method is practiced by: (1) incubating one  
10 or more candidate compounds with cells transfected with a DNA sequence encoding at least a portion of a morphogen non-coding DNA sequence that is responsive to a Pax homeobox gene and which is competent to act on and affect expression of a reporter gene with which it is operatively associated; (2) measuring the level of reporter gene expression in the transfected cell, and (3) comparing the level of reporter gene expressed in the presence of the candidate  
15 compound with the level of reporter gene expressed in the absence of the candidate compound. The level of an expressed reporter gene product in a given cell culture, or a change in that level resulting from exposure to one or more compound(s) indicates that the compound can also modulate the level of the morphogen normally associated with the non-coding sequence. Specifically, an increase in the level of reporter gene expression is indicative of a candidate  
20 compound's ability also to increase morphogen expression *in vivo*. Similarly, a decrease in the level of reporter gene expression is indicative of a candidate compound's ability also to decrease or otherwise interfere with morphogen expression *in vivo*. The above method is particularly useful for identifying compounds that are capable of influencing Pax mediated OP-1 gene expression.

25 The methods of the invention can therefore be used to identify compounds showing promise as therapeutics for various *in vivo* and *ex vivo* mammalian applications, as well as to identify compounds having numerous utilities. For example, compounds that modulate morphogen expression by stimulating Pax 2 or Pax 6 mediated transcription of a morphogen can be used *in vivo* to correct or alleviate a disease condition, to regenerate lost or damaged tissue, to

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induce cell proliferation and differentiation, and/or to maintain cell and tissue viability and/or a differentiated phenotype *in vivo* or *ex vivo*. The compounds also can be used to maintain the viability of, and the differentiated phenotype of, cells in culture. The various *in vivo*, *ex vivo*, and *in vitro* utilities and applications of the morphogenic proteins described herein are well

5 documented in the art. See, for example, US 92/01968 (WO 94/03200), filed March 11, 1992; US 92/07358 (WO 93/04692), filed August 28; PCT US 92/0743 (WO 93/05751), filed August 28, 1992; US 93/07321 (WO 94/03200), filed July 29, 1993; US 93/08808 (WO 94/06449), filed September 16, 1993; US93/08885 (WO94/06420), filed September 15, 1993, and US Pat. No. 5,266,683.

10 In another aspect, the invention further provides vectors and cells useful for morphogen, particularly OP-1, therapy. In one embodiment, the invention features a vector having a reporter gene operatively associated with at least a portion of one or more OP-1 non-coding sequences responsive to Pax homeobox gene products. The OP-1 non-coding sequences comprise at least a first Pax responsive OP-1 modulating element which is responsive to a first Pax gene expression  
15 product. In other embodiments, vectors further comprise a second non-coding sequence comprising at least a second Pax-responsive OP-1 modulating element which is responsive to a second Pax gene expression product; in such embodiments, the first and second Pax gene expression product differ. OP-1 non-coding sequences which are Pax responsive OP-1 modulating elements can be selected from nucleotides 1-3317 of SEQ. ID No. 1. Also  
20 anticipated to be similarly useful are certain of the non-coding sequences of other species homologues of OP-1 and proteins closely related to OP-1. For example, other non-coding DNA that is responsive to Pax gene products or homologues thereof can be used to identify modulators of specific morphogens, or other factors capable of modulating morphogen gene expression.

In another embodiment, the vector can include a non-coding OP-1-specific sequence  
25 selected from at least one of the following sequence segments of SEQ. ID No. 1 presented below, which defines approximately 3.3 Kb of 5' non-coding human genomic OP-1 sequence. Preferred vectors comprise sequence segments including nucleotides 1-3317, as well as shorter fragments of this region of DNA such as approximately nucleotides 108-121, 139-154, 157-167, 365-378, 491-503, 598-613, 737-747, 891-903, 994-1006, 1123-1140, 1144-1161, 1285-1297, 1750-1762,  
30 2001-2023, 2365-2378, 2931-2944 of SEQ. ID No. 1, including allelic, species and other

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sequence variants thereof. As base 2790 is the mRNA start site, other preferred sequences include approximately 2790-3317, representing transcribed but not translated 5' non-coding sequence and shorter fragments of this DNA region. Other preferred regions of the 5' non-coding region of SEQ. ID No. 1 include regions comprising a cluster of several Pax responsive elements, such as, for example, approximately 1-2073, 1-1297, 1-2691, 1-378, 491-1006, 1750-2023, 1750-2378, 1750-2691, 1750-2944. In certain embodiments, non-coding sequences correspond to part or all of SEQ. ID No. 2 and/or SEQ. ID No. 3, including allelic, species and other sequence variants thereof. In yet other embodiments, vectors comprise non-coding sequences corresponding to at least one, preferably between one and twelve and/or four or more first and second Pax-responsive OP-1 modulating elements, respectively. First Pax-responsive sites correspond approximately to bases 108-121, 139-154, 157-167, 365-378, 497-511, 598-613, 1123-1140, 1144-1161, 1285-1297, 1750-1762, 2001-2023, 2365-2378 and 2931-2944 of SEQ. ID No. 1. Second Pax-responsive sites correspond approximately to bases 491-503, 737-747, 891-903, and 994-1006 of SEQ. ID No. 1.

In another aspect, the invention provides a cell comprising a reporter gene whose regulation is mediated by one or more of the Pax-responsive OP-1 non-coding sequences defined above. In one embodiment, the cell is transfected with a reporter gene in operative association with at least one Pax responsive site. In another embodiment, the present invention provides a cell comprising a transfected vector encoding a reporter gene operatively associated with at least two DNA sequences, the first comprising at least part of a sequence selected from SEQ. ID No. 2 while the second comprises at least part of a sequence selected from SEQ. ID No. 3, including allelic, species and other sequence variants of the foregoing. In yet another embodiment, cells of the present invention are co-transfected with expression vectors encoding Pax gene expression products such as, for example, Pax 2 and/or Pax 6.

In another aspect, the invention provides kits useful in the design and/or identification of OP-1 expression modulating compounds. As used herein a "kit" comprises a cell comprising a reporter gene in operative association with an OP-1 non-coding DNA sequence and the reagents necessary for detecting expression of the reporter gene. The portion of OP-1 non-coding DNA chosen can be any of the various sequences which have been described herein above.

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Following this disclosure, medium flux screen assays, and kits therefore, for identifying modulators of morphogen expression, such as OP-1 expression, are available. These compounds can be naturally occurring molecules, or they can be designed and biosynthetically created using a rational drug design and an established structure/function analysis methodology. The compounds can be amino acid-based or can be composed in part or wholly of non-proteinaceous synthetic organic molecules.

The OP-1 expression modulating compounds thus identified can be produced in reasonable quantities, including commercially significant quantities, using standard recombinant expression or chemical synthesis technology well known and characterized in the art and/or as described herein. For example, automated means for the chemical synthesis of nucleic and amino acid sequences are commercially available. Alternatively, promising candidate compounds can be modified using standard biological or chemical methodologies to, for example, enhance the binding affinity of the compound for a DNA element and the preferred candidate compound derivative then can be produced in quantity.

Once a candidate compound has been identified and produced, it can be further tested for its effect on OP-1 expression. For example, a compound which upregulates (increases) the production of OP-1 (e.g., an OP-1 agonist) in a kidney cell line is a candidate for systemic administration. The candidate compound can be assayed in an animal model to determine the candidate molecule's efficacy *in vivo*. For example, the ability of a compound to upregulate levels of circulating OP-1 *in vivo* can be used to correct bone metabolism diseases such as osteoporosis (See, for example, PCT/US92/07932, above). Conversely, compounds which down regulate (decrease) the production of OP-1 (e.g., OP-1 antagonists) are also contemplated to be useful. Useful *in vivo* animal models for systemic administration are disclosed in the art and below.

As is well known in the art, OP-1 is differentially expressed in different cell types. Accordingly, it is further anticipated that a candidate compound will have utility as an inducer of OP-1 expression in one cell type but not in another. Thus, the invention further contemplates testing a candidate compound for its utility in modulating expression of OP-1 in a tissue specific manner *in vivo*.

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Thus, in view of this disclosure, one of ordinary skill in recombinant DNA and tissue culture techniques can design and construct appropriate DNA vectors and transfect cells with appropriate DNA sequences for use in the method according to the invention to assay for compounds which modulate the expression of OP-1. These identified compounds can be used to modulate OP-1 production and its effective concentrations in both *in vivo* and *in vitro*.

### **Brief Description of the Drawings**

Figure 1A is a diagrammatical illustration of the 5' non-coding region of the OP-1 gene in operative association with a luciferase reporter gene. Certain preferred Pax consensus sites are depicted along the non-coding region.

Figure 1B is a graphical representation of the induction of luciferase activity in G401 human kidney cells transfected with OP-1 promoter deletion constructs of (1A) in the presence or absence of co-transfection with Pax 2 and/or Pax 6 expression constructs.

Figure 2 shows the relative levels of luciferase expression from constructs containing 5' non-coding regions of the OP-1 gene in operative association with a luciferase reporter gene.

Figure 3 shows the levels of luciferase expression in cells containing pAS3.3 and pAS3.3d.

### **Detailed Description**

As will be more fully described below, we have discovered specific regions in the OP-1 gene sequence useful in identifying molecules capable of modulating OP-1 expression *in vivo*.

We discovered DNA sequences responsible for the regulation of OP-1 gene expression by cloning and characterizing various truncated sequences isolated from the 5' non-coding sequences of the human OP-1 gene. The presence of sequences defining Pax 2 or Pax 6 consensus binding sequences as defined herein in the OP-1 non-coding region, together with the observed modulation of OP-1 gene expression mediated by Pax 2 and Pax 6 gene products, implicate these sequences, or variants thereof, as having utility in a method for the screening of compounds for their ability to modulate OP-1 expression. Moreover, Pax responsive sequences

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located in 5' non-coding sequences of other morphogen genes provide a means for identifying compounds that modulate expression of other such morphogens.

### **List of Useful Terms and Definitions**

As used herein, "morphogen" means the class of proteins typified by human osteogenic protein 1 (hOP-1). hOP-1 and functionally equivalent morphogens are, as defined herein, dimeric proteins that induce or reinstate mammalian cells, particularly uncommitted progenitor cells, to undergo a fully integrated developmental cascade of cellular and molecular events that culminates in the formation of fully differentiated, functional tissue of a type appropriate to the context or local biological environment in which morphogenesis is induced, including any vascularization, connective tissue formation, enervation and the like characteristic of tissue naturally-occurring in such a context. For example, if cells are stimulated by OP-1 in the context of, for example, bone, liver, nerve, tooth dentin, periodontal tissue, gastrointestinal tract lining tissue, the resulting cascade of morphogenesis culminates in the formation of new or regenerative differentiated tissue appropriate to that local environment. Morphogenesis therefore differs significantly from simple reparative healing processes in which scar tissue (e.g., fibrous connective tissue) is formed and fills a lesion or other defect in differentiated, functional tissue.

Morphogens generally induce all of the following biological functions in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated cells; and supporting the growth and maintenance of differentiated cells. The term "progenitor cells" includes uncommitted cells, preferably of mammalian origin, that are competent to differentiate into one or more specific types of differentiated cells, depending on their genomic repertoire and the tissue specificity of the permissive environment in which morphogenesis is induced. Preferably, morphogenesis culminates in the formation of differentiated tissue having structural and functional properties of a tissue that occurs naturally in the body of a mammal. Morphogens further can delay or mitigate the onset of senescence- or quiescence-associated loss of phenotype and/or tissue function. Morphogens still further can stimulate phenotypic expression of differentiated cells, including expression of metabolic and/or functional, e.g., secretory, properties thereof. In addition, morphogens can induce redifferentiation of transformed

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cells under appropriate environmental conditions. As noted above, morphogens that induce proliferation and differentiation at least of mammalian bone progenitor cells, and/or support the formation, growth, maintenance and functional properties of mammalian endochondral bone tissue, are representative.

5 A morphogen as isolated from natural sources in mature, biologically active form is a glycosylated dimer typically having an apparent molecular weight of about 30-36 kDa as determined by SDS-PAGE. When reduced, the 30 kDa protein gives rise to two glycosylated peptide subunits having apparent molecular weights of about 16 kDa and 18 kDa. The reduced polypeptides themselves have no detectable morphogenic activity. Glycosylation, however, is  
10 not required for biological activity. The unglycosylated protein has an apparent molecular weight of about 27 kDa. When reduced, the 27 kDa protein gives rise to two unglycosylated polypeptides having molecular weights of about 14 kDa to 16 kDa. The polypeptides which together form the biologically active dimer comprise at least six, preferably at least seven, positionally conserved cysteine residues as set forth in U.S.S.N. 08/396,930, the teachings of  
15 which have been incorporated herein by reference. As described above, particularly preferred sequences include those comprising the C-terminal 96 or 102 amino acid sequences of DPP (from *Drosophila*), Vgl (from *Xenopus*), Vgr-1 (from mouse), the OP1 and OP2 proteins, proteins (see U.S. Pat. No. 5,011,691 and Oppermann *et al.*, as well as the proteins referred to as BMP2, BMP3, BMP4 (see WO88/00205, U. S. Patent No. 5,013,649 and W091/18098), BMP5  
20 and BMP6 (see WO90/11366, PCT/US90/01630), BMP8 and BMP9.

As stated above, the representative morphogen, for purposes of the present invention, comprises an OP-1 or an OP-1-related polypeptide. Sequences of useful OP-1 polypeptides are recited in US Pat Nos. 5,011,691; 5,018,753 and 5,266,683; in Ozkaynak *et al.* (1990) *EMBO J* 9:2085-2093; and Sampath *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 6004-6008. Additional  
25 useful sequences occur in the C-terminal domains of DPP (from *Drosophila*), Vgl (from *Xenopus*), 60A (from *Drosophila*, see Wharton *et al.* (1991), *Proc. Natl. Acad. Sci. USA* 88:9214-9218), Vgr-1 (from mouse), the OP-1 and OP2 proteins, (see U.S. Pat. No. 5,011,691 by Oppermann *et al.*), as well as the proteins referred to as BMP2, BMP3, BMP4 (see WO88/00205, U. S. Patent No. 5,013,649 and W091/18098), BMP5 and BMP6 (see WO90/11366,  
30 PCT/US90/01630) and BMP8 and 9. Each of the foregoing polypeptides, when oxidized and

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dimerized, is useful as a morphogen herein. Further, this family of morphogenic proteins includes longer forms of a given protein, as well as phylogenetic, e.g., species and allelic variants and biosynthetic mutants thereof, including addition and deletion mutants and variants, such as those which may alter the conserved C-terminal cysteine skeleton, provided that the alteration still allows the protein to form a dimeric species having a conformation capable of inducing morphogenesis, e.g., endochondral bone formation when implanted in a mammal in conjunction with a matrix permissive of bone morphogenesis. In addition, morphogens as defined herein can include forms having varying glycosylation patterns and varying N-termini, can be naturally occurring or biosynthetically derived, and can be produced by expression of recombinant DNA in prokaryotic or eukaryotic host cells according to established techniques. The proteins are active either as homodimers or heterodimers.

As used herein, the terms "morphogen", "bone morphogen", "bone morphogenic protein", "BMP", "osteogenic protein" and "osteogenic factor" embrace the class of proteins typified by human osteogenic protein 1 (hOP-1). It will be appreciated by the artisan of ordinary skill in the art, however, that OP-1 merely is representative of the TGF- $\beta$  subclass of true tissue morphogens competent to act as osteogenic proteins, and is not intended to limit the description. Morphogenic protein is generally understood to mean a protein which can induce the full cascade of morphogenic events culminating in at least endochondral bone formation. Other known, and useful proteins include, OP2, OP3, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-15, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, Vg1, Vgr, 60A, DPP, NODAL, UNIVIN, SCREW, ADMP, NEURAL and morphogenically active amino acid variants thereof. As defined herein, morphogenic proteins include biologically active species variants of any of these proteins, including conservative amino acid sequence variants, proteins encoded by degenerate nucleotide sequence variants, and morphogenically active proteins sharing the conserved seven cysteine skeleton as defined herein and encoded by a DNA sequence competent to hybridize to a DNA sequence encoding an osteogenic protein disclosed herein, including, without limitation, OP-1, BMP-5, BMP-6, BMP-2, BMP-4 or GDF-5, GDF-6 or GDF-7. Morphogenic proteins include those sharing the conserved seven cysteine domain and sharing at least 70% amino acid sequence homology (similarity) within the C-terminal active domain, as defined herein. That is, particularly preferred morphogenic proteins are those comprising an

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amino acid sequence having at least 70% homology with the C-terminal 102-106 amino acids, defining the conserved seven cysteine domain, of human OP-1 and related proteins. Certain preferred embodiments of the instant invention relate to the morphogenic protein, OP-1. "Amino acid sequence homology" is understood herein to mean amino acid sequence similarity.

5 Homologous sequences share identical or similar amino acid residues, where similar residues are conservative substitutions for, or allowed point mutations of, corresponding amino acid residues in an aligned reference sequence. Thus, a candidate polypeptide sequence that shares 70% amino acid homology with a reference sequence is one in which any 70% of the aligned residues are either identical to, or are conservative substitutions of, the corresponding residues in a reference  
10 sequence. Examples of conservative variations include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine or methionine, for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid, provided that  
15 antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, *e.g.*, substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; phenylalanine, and tyrosine.

20 Naturally occurring proteins identified and/or appreciated herein to be morphogenic proteins form a distinct subgroup within the loose evolutionary grouping of sequence-related proteins known as the TGF- $\beta$  superfamily or supergene family. The naturally occurring morphogens share substantial amino acid sequence homology in their C-terminal regions (domains). Morphogenic proteins comprise a pair of polypeptides with amino acid sequences  
25 each comprising a sequence that shares a defined relationship with an amino acid sequence of a reference morphogen. Herein, preferred morphogenic polypeptides share a defined relationship with a sequence present in active human OP-1. However, any one or more of the naturally occurring or biosynthetic sequences disclosed herein similarly could be used as a reference sequence. Preferred osteogenic polypeptides share a defined relationship with at least the  
30 C-terminal six cysteine domain of human OP-1. Preferably, morphogenic polypeptides share a

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defined relationship with at least the C-terminal seven cysteine domain of human OP-1. That is, preferred polypeptides in a dimeric protein with morphogenic activity each comprise a sequence that corresponds to a reference sequence or is functionally equivalent thereto.

Functionally equivalent sequences include functionally equivalent arrangements of  
 5 cysteine residues disposed within the reference sequence, including amino acid insertions or deletions which alter the linear arrangement of these cysteines, but do not materially impair their relationship in the folded structure of the dimeric morphogen protein, including their ability to form such intra- or inter-chain disulfide bonds as may be necessary for morphogenic activity. Functionally equivalent sequences further include those wherein one or more amino acid residues  
 10 differs from the corresponding residue of a reference sequence, *e.g.*, the C-terminal seven cysteine domain (also referred to herein as the conserved seven cysteine skeleton) of human OP-1, provided that this difference does not destroy morphogenic activity. Accordingly, conservative substitutions of corresponding amino acids in the reference sequence are preferred. Amino acid residues that are conservative substitutions for corresponding residues in a reference  
 15 sequence are those that are physically or functionally similar to the corresponding reference residues, *e.g.*, that have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Particularly preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff *et al.* (1978), *5 Atlas of Protein Sequence and Structure*, Suppl. 3, ch. 22 (pp. 354-352), Natl.  
 20 Biomed. Res. Found., Washington, D.C. 20007, the teachings of which are incorporated by reference herein.

Publications disclosing these sequences, as well as their chemical and physical properties, include: *OP-1 and OP-2*: U.S. 5,011,691, U.S. 5,266,683, Ozkaynak *et al.* (1990) *EMBO J.* 9: 2085-2093; *OP-3*: WO94/10203 (PCT US93/10520); *BMP2, BMP3, BMP4*: WO88/00205,  
 25 Wozney *et al.* (1988) *Science* 242: 1528-1534; *BMP5 and BMP6*: Celeste *et al.* (1991) *PNAS* 87: 9843-9847; *Vgr-1*: Lyons *et al.* (1989) *PNAS* 86: 4554-4558; *DPP*: Padgett *et al.* (1987) *Nature* 325: 81-84; *Vgr-1*: Weeks (1987) *Cell* 51: 861-867; *BMP-9*: WO95/33830  
 (PCT/US95/07084); *BMP10*: WO94/26893 (PCT/US94/05290); *BMP-11*: WO94/26892  
 (PCT/US94/05288); *BMP12*: WO95/16035 (PCT/US94/14030); *BMP-13*: WO95/16035  
 30 (PCT/US94/14030); *GDF-1*: WO92/00382 (PCT/US91/04096) and Lee *et al.* (1991) *PNAS* 88:

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4250-4254; *GDF-8*: WO94/21681 (PCT/US94/03019); *GDF-9*: WO94/15966  
(PCT/US94/00685); *GDF-10*: WO95/10539 (PCT/US94/11440); *GDF-11*: WO96/01845  
(PCT/US95/08543); *BMP-15*: WO96/36710 (PCT/US96/06540); *MP121*: WO96/01316  
(PCT/EP95/02552); *GDF-5 (CDMP-1, MP52)*: WO94/15949 (PCT/US94/00657) and  
5 WO96/14335 (PCT/US94/12814) and WO93/16099 (PCT/EP93/00350); *GDF-6 (CDMP-2,*  
*BMP13)*: WO95/01801 (PCT/US94/07762) and WO96/14335 and WO95/10635  
(PCT/US94/14030); *GDF-7 (CDMP-3, BMP12)*: WO95/10802 (PCT/US94/07799) and  
WO95/10635 (PCT/US94/14030). In another embodiment, useful proteins include biologically  
active biosynthetic constructs, including novel biosynthetic morphogenic proteins and chimeric  
10 proteins designed using sequences from two or more known morphogens. See also the  
biosynthetic constructs disclosed in U.S. Pat. 5,011,691, the disclosure of which is incorporated  
herein by reference (*e.g.*, COP-1, COP-3, COP-4, COP-5, COP-7, and COP-16).

As earlier stated, morphogenic proteins contemplated herein include those in which the  
amino acid sequences comprise a sequence sharing at least 70% amino acid sequence homology  
15 or "similarity", and preferably 80% homology or similarity, with a reference morphogenic  
protein selected from the foregoing naturally occurring proteins. Preferably, the reference  
protein is human OP-1, and the reference sequence thereof is the C-terminal seven cysteine  
domain present in osteogenically active forms of human OP-1. A polypeptide suspected of  
being functionally equivalent to a reference morphogen polypeptide is aligned therewith using  
20 the method of Needleman, *et al.* (1970) *J. Mol. Biol.* 48:443-453, implemented conveniently by  
computer programs such as the Align program (DNASTar, Inc.). As noted above, internal gaps  
and amino acid insertions in the candidate sequence are ignored for purposes of calculating the  
defined relationship, conventionally expressed as a level of amino acid sequence homology or  
identity, between the candidate and reference sequences. "Amino acid sequence homology" is  
25 understood herein to include both amino acid sequence identity and similarity. Homologous  
sequences share identical and/or similar amino acid residues, where similar residues are  
conservative substitutions for, or "allowed point mutations" of, corresponding amino acid  
residues in an aligned reference sequence. Thus, a candidate polypeptide sequence that shares  
70% amino acid homology with a reference sequence is one in which any 70% of the aligned  
30 residues are either identical to, or are conservative substitutions of, the corresponding residues in  
a reference sequence. In a currently preferred embodiment, the reference sequence is OP-1.

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Morphogenic proteins useful herein accordingly include allelic, phylogenetic counterpart and other variants of the preferred reference sequence, whether naturally-occurring or biosynthetically produced (*e.g.*, including "muteins" or "mutant proteins"), as well as novel members of the general morphogenic family of proteins, including those set forth and identified above. Certain particularly preferred morphogenic polypeptides share at least 60% amino acid identity with the preferred reference sequence of human OP-1, still more preferably at least 65% amino acid identity therewith.

As noted above, certain currently preferred morphogenic polypeptide sequences have greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the preferred reference sequence of hOP-1. These particularly preferred sequences include allelic and phylogenetic counterpart variants of the OP-1 and OP-2 proteins, including the *Drosophila* 60A protein. Accordingly, preferred morphogenic proteins include active proteins comprising pairs of polypeptide chains within the generic amino acid sequence herein referred to as "OPX" (see below and SEQ ID NO: 4), which defines the seven cysteine skeleton. As described therein, each Xaa at a given position independently is selected from the residues occurring at the corresponding position in the C-terminal sequence of mouse or human OP-1 or OP-2.

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Cys | Xaa | Xaa | His | Glu | Leu | Tyr | Val | Ser | Phe | Xaa | Asp | Leu | Gly | Trp | Xaa | Asp | Trp |     |
|    | 1   |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |     |     |     |     |
| 20 | Xaa | Ile | Ala | Pro | Xaa | Gly | Tyr | Xaa | Ala | Tyr | Tyr | Cys | Glu | Gly | Glu | Cys | Xaa | Phe | Pro |
|    | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |     | 35  |     |     |     |     |
|    | Leu | Xaa | Ser | Xaa | Met | Asn | Ala | Thr | Asn | His | Ala | Ile | Xaa | Gln | Xaa | Leu | Val | His | Xaa |
|    |     | 40  |     |     |     | 45  |     |     |     |     | 50  |     |     |     | 55  |     |     |     |     |
|    | Xaa | Xaa | Pro | Xaa | Xaa | Val | Pro | Lys | Xaa | Cys | Cys | Ala | Pro | Thr | Xaa | Leu | Xaa | Ala |     |
| 25 |     |     | 60  |     |     |     | 65  |     |     |     | 70  |     |     |     |     |     |     |     |     |
|    | Xaa | Ser | Val | Leu | Tyr | Xaa | Asp | Xaa | Ser | Xaa | Asn | Val | Ile | Leu | Xaa | Lys | Xaa | Arg |     |
|    | 75  |     |     | 80  |     |     |     | 85  |     |     |     | 90  |     |     |     |     |     |     |     |
|    | Asn | Met | Val | Val | Xaa | Ala | Cys | Gly | Cys | His |     |     |     |     |     |     |     |     |     |
|    |     | 95  |     |     |     | 100 |     |     |     |     |     |     |     |     |     |     |     |     |     |

wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 11 = (Arg or Gln); Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or

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Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr); Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr or His); and Xaa at res. 97 = (Arg or Lys).

5 In yet other preferred embodiments, morphogenic protein as contemplated herein can be further defined by a generic amino acid sequence. For example, SEQ. ID No. 5 and No. 6 disclosed herein represent composite amino acid sequences of the following proteins: human OP-1, human OP-2, human OP-3, human BMP-2, human BMP-3, human BMP-4, human BMP-5, human BMP-6, human BMP-8, human BMP-9, human BMP10, human BMP-11, 10 *Drosophila* 60A, *Xenopus* Vg-1, sea urchin UNIVIN, human CDMP-1 (mouse GDF-5), human CDMP-2 (mouse GDF-6, human BMP-13), human CDMP-3 (mouse GDF-7, human BMP-12), mouse GDF-3, human GDF-1, mouse GDF-1, chicken DORSALIN, dpp, *Drosophila* SCREW, mouse NODAL, mouse GDF-8, human GDF-8, mouse GDF-9, mouse GDF-10, human GDF-11, mouse GDF-11, human BMP-15, and rat BMP3b. SEQ. ID NO: 5 accommodates the C-terminal 15 six cysteine skeleton and, SEQ. ID NO: 6 accommodates the seven cysteine skeleton.

## SEQ. ID NO: 5

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|    | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa |
|    | 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |
| 20 | Pro | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Cys | Xaa | Gly | Xaa | Cys | Xaa | Xaa | Xaa |
|    |     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |
|    | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa |
|    |     |     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     | 50  |
|    | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Cys | Xaa | Pro | Xaa | Xaa |
|    |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     | 65  |     |
| 25 | Xaa | Xaa | Xaa | Xaa | Xaa | Leu | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa |
|    |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |     |
|    | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Xaa | Cys | Xaa | Cys | Xaa |     |     |     |
|    |     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |

wherein each Xaa is independently selected from a group of one or more specified amino acids 30 defined as follows: "Res. " means "residue" and Xaa at res. 1 = (Phe, Leu or Glu); Xaa at res. 2 = (Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu); Xaa at res. 3 = (Val, Ile, Leu or Asp); Xaa at res. 4 = (Ser, Asp, Glu, Asn or Phe); Xaa at res. 5 = (Phe or Glu); Xaa at res. 6 = (Arg, Gln, Lys, Ser, Glu, Ala or Asn); Xaa at res. 7 = (Asp, Glu, Leu, Ala or Gln); Xaa at res. 8 = (Leu, Val, Met,

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Ile or Phe); Xaa at res. 9 = (Gly, His or Lys); Xaa at res. 10 = (Trp or Met); Xaa at res. 11 = (Gln, Leu, His, Glu, Asn, Asp, Ser or Gly); Xaa at res. 12 = (Asp, Asn, Ser, Lys, Arg, Glu or His); Xaa at res. 13 = (Trp or Ser); Xaa at res. 14 = (Ile or Val); Xaa at res. 15 = (Ile or Val); Xaa at res. 16 = (Ala, Ser, Tyr or Trp); Xaa at res. 18 = (Glu, Lys, Gln, Met, Pro, Leu, Arg, His or Lys); Xaa at res. 19 = (Gly, Glu, Asp, Lys, Ser, Gln, Arg or Phe); Xaa at res. 20 = (Tyr or Phe); Xaa at res. 21 = (Ala, Ser, Gly, Met, Gln, His, Glu, Asp, Leu, Asn, Lys or Thr); Xaa at res. 22 = (Ala or Pro); Xaa at res. 23 = (Tyr, Phe, Asn, Ala or Arg); Xaa at res. 24 = (Tyr, His, Glu, Phe or Arg); Xaa at res. 26 = (Glu, Asp, Ala, Ser, Tyr, His, Lys, Arg, Gln or Gly); Xaa at res. 28 = (Glu, Asp, Leu, Val, Lys, Gly, Thr, Ala or Gln); Xaa at res. 30 = (Ala, Ser, Ile, Asn, Pro, Glu, Asp, Phe, Gln or Leu); Xaa at res. 31 = (Phe, Tyr, Leu, Asn, Gly or Arg); Xaa at res. 32 = (Pro, Ser, Ala or Val); Xaa at res. 33 = (Leu, Met, Glu, Phe or Val); Xaa at res. 34 = (Asn, Asp, Thr, Gly, Ala, Arg, Leu or Pro); Xaa at res. 35 = (Ser, Ala, Glu, Asp, Thr, Leu, Lys, Gln or His); Xaa at res. 36 = (Tyr, His, Cys, Ile, Arg, Asp, Asn, Lys, Ser, Glu or Gly); Xaa at res. 37 = (Met, Leu, Phe, Val, Gly or Tyr); Xaa at res. 38 = (Asn, Glu, Thr, Pro, Lys, His, Gly, Met, Val or Arg); Xaa at res. 39 = (Ala, Ser, Gly, Pro or Phe); Xaa at res. 40 = (Thr, Ser, Leu, Pro, His or Met); Xaa at res. 41 = (Asn, Lys, Val, Thr or Gln); Xaa at res. 42 = (His, Tyr or Lys); Xaa at res. 43 = (Ala, Thr, Leu or Tyr); Xaa at res. 44 = (Ile, Thr, Val, Phe, Tyr, Met or Pro); Xaa at res. 45 = (Val, Leu, Met, Ile or His); Xaa at res. 46 = (Gln, Arg or Thr); Xaa at res. 47 = (Thr, Ser, Ala, Asn or His); Xaa at res. 48 = (Leu, Asn or Ile); Xaa at res. 49 = (Val, Met, Leu, Pro or Ile); Xaa at res. 50 = (His, Asn, Arg, Lys, Tyr or Gln); Xaa at res. 51 = (Phe, Leu, Ser, Asn, Met, Ala, Arg, Glu, Gly or Gln); Xaa at res. 52 = (Ile, Met, Leu, Val, Lys, Gln, Ala or Tyr); Xaa at res. 53 = (Asn, Phe, Lys, Glu, Asp, Ala, Gln, Gly, Leu or Val); Xaa at res. 54 = (Pro, Asn, Ser, Val or Asp); Xaa at res. 55 = (Glu, Asp, Asn, Lys, Arg, Ser, Gly, Thr, Gln, Pro or His); Xaa at res. 56 = (Thr, His, Tyr, Ala, Ile, Lys, Asp, Ser, Gly or Arg); Xaa at res. 57 = (Val, Ile, Thr, Ala, Leu or Ser); Xaa at res. 58 = (Pro, Gly, Ser, Asp or Ala); Xaa at res. 59 = (Lys, Leu, Pro, Ala, Ser, Glu, Arg or Gly); Xaa at res. 60 = (Pro, Ala, Val, Thr or Ser); Xaa at res. 61 = (Cys, Val or Ser); Xaa at res. 63 = (Ala, Val or Thr); Xaa at res. 65 = (Thr, Ala, Glu, Val, Gly, Asp or Tyr); Xaa at res. 66 = (Gln, Lys, Glu, Arg or Val); Xaa at res. 67 = (Leu, Met, Thr or Tyr); Xaa at res. 68 = (Asn, Ser, Gly, Thr, Asp, Glu, Lys or Val); Xaa at res. 69 = (Ala, Pro, Gly or Ser); Xaa at res. 70 = (Ile, Thr, Leu or Val); Xaa at res. 71 = (Ser, Pro, Ala, Thr, Asn or Gly); Xaa at res. 2 = (Val, Ile, Leu or Met); Xaa at res. 74 = (Tyr, Phe, Arg, Thr, Tyr or Met); Xaa at res. 75 = (Phe, Tyr, His, Leu, Ile, Lys, Gln or Val); Xaa

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at res. 76 = (Asp, Leu, Asn or Glu); Xaa at res. 77 = (Asp, Ser, Arg, Asn, Glu, Ala, Lys, Gly or Pro); Xaa at res. 78 = (Ser, Asn, Asp, Tyr, Ala, Gly, Gln, Met, Glu, Asn or Lys); Xaa at res. 79 = (Ser, Asn, Glu, Asp, Val, Lys, Gly, Gln or Arg); Xaa at res. 80 = (Asn, Lys, Thr, Pro, Val, Ile, Arg, Ser or Gln); Xaa at res. 81 = (Val, Ile, Thr or Ala); Xaa at res. 82 = (Ile, Asn, Val, Leu, Tyr, Asp or Ala); Xaa at res. 83 = (Leu, Tyr, Lys or Ile); Xaa at res. 84 = (Lys, Arg, Asn, Tyr, Phe, Thr, Glu or Gly); Xaa at res. 85 = (Lys, Arg, His, Gln, Asn, Glu or Val); Xaa at res. 86 = (Tyr, His, Glu or Ile); Xaa at res. 87 = (Arg, Glu, Gln, Pro or Lys); Xaa at res. 88 = (Asn, Asp, Ala, Glu, Gly or Lys); Xaa at res. 89 = (Met or Ala); Xaa at res. 90 = (Val, Ile, Ala, Thr, Ser or Lys); Xaa at res. 91 = (Val or Ala); Xaa at res. 92 = (Arg, Lys, Gln, Asp, Glu, Val, Ala, Ser or Thr); Xaa at res. 93 = (Ala, Ser, Glu, Gly, Arg or Thr); Xaa at res. 95 = (Gly, Ala or Thr); Xaa at res. 97 = (His, Arg, Gly, Leu or Ser). Further, after res. 53 in rBMP3b and mGDF-10 there is an Ile; after res. 54 in GDF-1 there is a T; after res. 54 in BMP3 there is a V; after res. 78 in BMP-8 and Dorsalin there is a G; after res. 37 in hGDF-1 there is Pro, Gly, Gly, Pro.

SEQ ID NO: 6 includes all of SEQ ID NO: 5 and in addition includes the following sequence (SEQ ID NO: 7) at its N-terminus:

#### SEQ ID NO: 7

|     |     |     |     |     |
|-----|-----|-----|-----|-----|
| Cys | Xaa | Xaa | Xaa | Xaa |
| 1   |     |     |     | 5   |

Accordingly, beginning with residue 6, each "Xaa" in SEQ. ID No. 6 is a specified amino acid defined as for SEQ. ID No. 5, with the distinction that each residue number described for SEQ. ID No. 5 is shifted by five in SEQ. ID No. 6. Thus, "Xaa at res. 1 = (Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu)" in SEQ. ID No. 5 refers to Xaa at res. 6 in SEQ. ID No. 6. In SEQ. ID No. 6, Xaa at res. 2 = (Lys, Arg, Gln, Ser, His, Glu, Ala, or Cys); Xaa at res. 3 = (Lys, Arg, Met, Lys, Thr, Leu, Tyr, or Ala); Xaa at res. 4 = (His, Gln, Arg, Lys, Thr, Leu, Val, Pro, or Tyr); and Xaa at res. 5 = (Gln, Thr, His, Arg, Pro, Ser, Ala, Gln, Asn, Tyr, Lys, Asp, or Leu).

Still further, morphogenically active proteins have polypeptide chains with amino acid sequences comprising a sequence encoded by a nucleic acid that hybridizes, under low, medium or high stringency hybridization conditions, to DNA or RNA encoding reference morphogen sequences, *e.g.*, C-terminal sequences defining the conserved seven cysteine domains of OP-1,

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OP-2, BMP2, BMP4, BMP5, BMP6, 60A, GDF3, GDF6, GDF7 and the like. As used herein, high stringency hybridization conditions are defined as hybridization according to known techniques in 40% formamide, 5 X SSPE, 5 X Denhardt's Solution, and 0.1% SDS at 37°C overnight, and washing in 0.1 X SSPE, 0.1% SDS at 50°C. Standard stringency conditions are well characterized in commercially available, standard molecular cloning texts. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D.N. Glover ed., 1985); *Oligonucleotide Synthesis* (M.J. Gait ed., 1984); *Nucleic Acid Hybridization* (B. D. Hames & S.J. Higgins eds. 1984); and B. Perbal, *A Practical Guide To Molecular Cloning* (1984).

As used herein, "gene expression" is understood to refer to the production of a gene product encoded by a DNA sequence of interest, including the transcription of the DNA sequence and/or translation of the mRNA transcript. It is understood that gene expression in the context of the present invention is not necessarily a consequence of direct interaction of specific proteins or factors with specific DNA sequences of the present invention, but that the reporter gene product is produced as a consequence of an inductive effect that is mediated or influenced by the presence and/or expression of Pax genes in the cells of the invention.

As used herein, "operative association" relates to a fusion of the described non-coding DNA sequences with a reporter gene in such a reading frame as to be co-transcribed, or at such a relative positioning as to be competent to modulate expression of the reporter gene.

As used herein, "vector" is understood to mean any nucleic acid comprising a nucleotide sequence of interest and competent to be incorporated into a host cell and capable of functional transcription. Such vectors include linear or circular nucleic acids, plasmids, phagemids, cosmids, YACs (yeast artificial chromosomes) and the like. Vector as used herein can be used to construct a test cell, or construct DNAs, cells and/or viruses suitable for gene therapy applications.

As used herein, "non-coding sequence" or "non-coding DNA" includes DNA sequences that are not transcribed into RNA sequence, and/or RNA sequences that are not translated into protein. This category of "non-coding sequence" has been defined for ease of reference in the

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application, and includes sequences occurring 5' to the ATG site of the OP-1 gene at nucleotide 3318 of SEQ. ID No. 1. Further, as used herein "5' non-coding sequence" or "5' non-coding DNA" define a gene locus upstream of or 5' to the translation start site of a reporter gene. In contrast, coding sequence means a translated and/or transcribed DNA sequence encoding a reporter gene or morphogenic protein as defined herein.

As used herein, an "OP-1-specific" or "morphogen-specific" non-coding sequence is understood to define a non-coding sequence that lies contiguous to an OP-1-specific or morphogen specific coding sequence at a gene locus under naturally-occurring conditions.

"Responsive," as defined herein, is not limited to direct interaction between elements of the invention (e.g. Pax responsive OP-1 modulating element and Pax gene products) but includes indirect effects on gene transcription or translation, for example, that are mediated or influenced by the Pax gene products.

As used herein, a "Pax responsive OP-1 modulating element" is a DNA element that is responsive to the presence of a Pax gene and/or expression of a Pax gene product. That is, expression of a gene operatively associated therewith is modulated when present in a cell together with DNA encoding a Pax gene. For example, when a reporter gene and Pax gene are co-transfected into a host cell, reporter gene expression is modulated by the presence and/or expression of an endogenous Pax gene. As discussed herein, it is contemplated that a Pax responsive modulating element(s) can be located in the 5' non-coding sequences of any morphogen as defined herein. Thus, for purposes of the present invention, a Pax responsive morphogen modulating element comprises at least all of the generic features (structural, functional, chemical) associated with a Pax responsive OP-1 modulating element, and further includes allelic, species and mutant variants thereof. A "Pax gene product" is a nucleic acid or protein product encoded by a gene which is a member of the Pax family of genes.

"Pax genes" encode a family of developmentally regulated transcription factors that have been implicated in a number of human and murine congenital disorders, as well as in tumorigenesis. These genes, also characterized as "paired box genes," are defined by the presence of an evolutionarily conserved DNA binding domain, termed the "paired domain". As

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demonstrated below in Example 2, Pax genes and/or their expression and/or transcription products are morphogen modulators as that group of compounds is defined herein.

Paired box genes were first identified in *Drosophila* as a family of related genes encoding a 128-amino acid DNA binding domain, and are expressed in temporally and spatially restricted patterns during development. The phenotypes associated with Pax gene mutations demonstrate that these gene products are critical during organogenesis. Missense mutation within the paired domain of Pax genes have been associated with congenital disorders in both mouse and man. Also, Pax genes have been demonstrated to have oncogenic potential, and, for example, a translocation involving the paired box portion of Pax 3 has been associated with a human tumor.

The consensus binding sequences for Pax 6 and Pax 2 paired domains are very similar. The Pax 6 consensus spans 20 base pairs and shares a central 10-base pair region of homology with the Pax 2 consensus consisting of TCACGC-TGA, where the dash indicates a nucleotide difference between the two sequences in this central base pair region (Epstein et al. (1994) *J. Biol. Chem.* 269:8355-8361, the disclosure of which is incorporated by reference herein).

The respective purified paired domain proteins exhibit high affinity for their respective consensus sequences. The Pax-6 paired domain has been shown to bind to this consensus sequence as a monomer. The paired domain, when binding to such a DNA sequence, adopts an  $\alpha$ -helical conformation and contacts residues within a large span of the DNA molecule.

As used herein, a "Pax 2 consensus binding sequence" or "Pax 2 consensus binding element" is a nucleotide sequence which has been shown to be bound by the DNA binding protein Pax 2. The consensus sequence of the Pax 2 binding site has been determined by homology to be: G T C A C/T G C G/A T G A, as depicted in SEQ. ID No. 3. (Epstein et al. (1994) *J. Biol. Chem.* 269:8355-8361). A "Pax 6 consensus binding sequence" or "Pax 6 consensus binding element" is a nucleotide sequence which has been shown to be bound by the DNA binding protein Pax 6. The consensus sequence of the Pax 6 binding site has been determined by homology to be T T C A C G C A/T T G/C A N T G/T A/C N T/C, as depicted in SEQ. ID No. 2 where N=A, G, C or T. (Epstein et al. (1994) *J. Biol. Chem.* 269:8355-8361.) Another suitable consensus sequence for purposes of the present invention is A--

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TTCACGCATGA-T or TTCACGCATGA, wherein the dashes indicate that any nucleotide can reside at that locus.

“Pax 2 or Pax 6 mediated transcription or expression” means transcription or expression of a reporter gene operatively associated with a Pax responsive modulating element, or functional  
5 equivalent thereof, as described elsewhere herein. Such transcription or expression occurs in the presence of Pax genes and/or Pax gene expression products, but their presence is not required.

“Pax 2 or Pax 6 mediated transcription or expression” can involve, for example, the direct physical interaction of Pax 2 or Pax 6 gene products with the Pax 2 or Pax 6 consensus binding sequences, as defined herein above. Alternatively, “Pax mediated transcription or expression”  
10 can be modulated by compounds that can mimic the transcription or expression events otherwise associated with Pax family proteins. Such compounds are contemplated herein generally as morphogen modulators, and more specifically as Pax analogs.

As discussed elsewhere herein, “allelic, species and other sequence variants thereof” includes point mutations, insertions and deletions such as those that are naturally occurring or  
15 which can be genetically engineered into an OP-1 non-coding DNA sequence using routine methods and which do not substantially affect the regulation of a reporter gene by the OP-1 non-coding sequence. For example, one of ordinary skill in the art can use site directed mutagenesis to modify, as by deletion, for example, one or more of the OP-1 non-coding sequences described herein without substantially affecting the regulation of OP-1 or a reporter gene by the  
20 modification. Such modifications are considered to be within the scope of the disclosure provided herein.

As discussed elsewhere herein, “analog” or “variant” is intended to mean a DNA or a protein which mimics or performs similar functions relative to a naturally-occurring or representative DNA or protein. For example, a DNA that is responsive to Pax 2 or Pax 6 in the  
25 same manner as those described herein, yet are structurally different therefrom, are considered DNA analogs or DNA variants within the scope of this invention. Similarly, any expression product which can modulate OP-1 expression in the same manner as Pax 2 and/or Pax 6 as disclosed herein, are considered Pax 2 and/or Pax 2 analogs or variants within the scope of this invention.

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"Co-transfection" refers to the simultaneous or sequential transfection of two or more vectors into a given cell.

Where a cell line is to be established, particularly where the transfected DNA is to be incorporated into the cell's genome, lines that can be immortalized are especially desirable. As used herein, "immortalized" cell lines are viable for multiple passages without significant reduction in growth rate or protein production. It is contemplated that the cells of the present invention have utility for cell mediated gene therapy, where cells producing high levels of a reporter gene such as OP-1 are desired. It is further contemplated that the Pax consensus binding sequences or other useful sequences can be altered or adapted for viral mediated gene therapy using routine methods.

The Pax genes and/or gene products as used herein are capable of stimulating the transcription of a morphogen gene and are herein also referred to as "morphogen stimulators", "morphogen modulating compounds" or "OP-1 modulating compounds."

A "candidate compound" is any test substance that can be used to treat the cells of the present invention *in vitro*, *in vivo* or *ex vivo* to determine their usefulness as OP-1 modulating compounds. Accordingly, and in keeping with the earlier definition, a candidate compound which can modulate OP-1 expression in the manner of Pax 2 and/or Pax 6 as disclosed herein, is also a morphogen stimulator as well as a Pax 2 and/or Pax 6 analog. Conversely, a candidate compound which acts contrary to Pax 2 and/or Pax 6 as disclosed herein, is a morphogen suppressor or Pax 2/Pax 6 antagonist.

#### **The OP-1 upstream region contains a silencer of gene expression**

The presence of a sequence within the OP-1 upstream region that reduces OP-1 gene expression (a silencer fragment) was identified in expression assays described below in Example 9. The approximately 0.1 kb AatII-PvuII fragment (approximately nucleotides 2606-2690 of SEQ ID NO: 1) reduces OP-1 expression by approximately three fold (between approximately two fold and five fold in the experiments described in Example 9).

In a preferred embodiment of the invention, expression of OP-1 is increased by removing the silencer fragment from the OP-1 upstream region. In one embodiment, the silencer fragment

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is removed from a plasmid or other recombinant construct containing the OP-1 gene. In an alternative embodiment, a recombinant cell line is made wherein one or both genomic copies of the OP-1 gene are modified to remove the silencer fragment, using methods known in the art.

In another embodiment of the invention, the silencer fragment is used to modulate the expression of another (non-OP-1) gene. One or more copies of the silencer fragment are inserted in the promoter region of the gene in order to reduce expression. The silencer fragment is inserted into the promoter region of a gene that is contained in a plasmid, a recombinant vector, or in the genome itself.

The silencer fragment contained within the AatII-PvuII fragment can be more precisely identified by standard deletion analysis of the fragment using the expression assay described in Examples 2 and 9. Truncated silencer fragments, identified by a further deletion analysis, are also useful in the invention.

A naturally occurring sequence variant of the silencer fragment (or of a truncated silencer fragment), or a nucleic acid which hybridizes to the silencer fragment (or to a truncated silencer fragment) under high stringency hybridization conditions, is also useful in the invention.

#### **Exemplary Cells, Vectors, Reporter Genes and Assays For Use In Screening Compounds Which Modulate OP-1 Gene Expression**

##### **A. Useful Cells**

Any eukaryotic cell, including an immortalized cell line suitable for long term culturing conditions, is contemplated to be useful for the methods and cells of the invention. Useful cells should be easy to transfect, are capable of stably maintaining foreign DNA with an unarranged sequence, and have the necessary cellular components for efficient transcription and translation of the protein, including any elements required for post-translational modification and secretion, if necessary. Where the cell is to be transfected with a non-dominating selection gene, the cell genotype preferably is deficient for the endogenous selection gene. Preferably, the cell line also has simple media composition requirements, and rapid generation times. Useful cell lines are mammalian cell lines, including myeloma, HeLa, fibroblast, embryonic and various tissue cell lines, e.g., kidney, liver, lung and the like. The cells may be derived from tissue or subcultured

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from established cell lines. As used herein, "derived" means the cells are from the cultured tissue itself or are a cell line whose parent cells are of the tissue itself. Cell lines particularly useful in practicing the present invention are, for example, Y79 human retinoblastoma cells, G401 human kidney cells, ROS human osteoblastic cells, MCF-7 human mammary cancer cells and LLCBK1 porcine proximal tubule cells. A large number of cell lines now are available through the American Type Culture Collection (Rockville, MD) or through the European Collection of Animal Cell Cultures (Porton Down, Salisbury, SP4 0JG, U.K.)

Where the expression of a reporter gene that is controlled by non-coding sequences of the morphogen OP-1 is to be analyzed, particularly useful cells and cell lines are envisioned to include eukaryotic, preferably mammalian cells of a tissue and cell type known to express OP-1 and/or closely related proteins. See, for example, Ozkaynak, et al. (1991), *Biochem. Biophys. Res. Commun.* 179: 116-123 for a detailed description of tissues known to express OP-1. Such cells, include, without limitation, cells of uro-genital cell origin, including kidney, bladder and ovary cells, lung, liver, bone, nerve, mammary gland and cardiac cells, cells of gonadal origin, cells of gastrointestinal origin, glial cells and other cell lines known to express endogenous genes encoding morphogenic proteins. Preferred cell lines are of epithelial origin.

Cell cultures of kidney, adrenals, urinary bladder, brain, or other organs, can be prepared as described widely in the literature. For example, kidneys can be explanted from neonatal or new born or young or adult rodents (mouse or rat) and used in organ culture as whole or sliced (1-4 mm) tissues. Primary tissue cultures and established cell lines, also derived from kidney, adrenals, urinary, bladder, brain, mammary, or other tissues can be established in multiwell plates (6 well or 24 well) according to conventional cell culture techniques, and are cultured in the absence or presence of serum for a period of time (1-7 days). Cells can be cultured, for example, in DMEM containing 10% fetal calf serum or in serum-deprived medium or in defined medium (e.g., containing insulin, transferrin, glucose, albumin, or other growth factors). Test compounds are added to the cultured cells and OP-1 biosynthesis monitored and measured at various time points using the methods described previously herein. Suitable cell lines include cell lines that have been shown to contain high levels of OP-1 mRNA, indicating that the OP-1 promoter is active in the cells. Cells and their culture fluids are assayed using techniques well known in the art, either for mRNA levels, using Northern blot analysis and OP-1 mRNA specific

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probes, or for protein levels, using OP-1 specific antibodies. For example, OP-1 protein can be measured on a tissue section or cell directly using standard immunofluorescence techniques or in culture fluids and body fluids using a sandwich immunoassay. Other methods for detecting, measuring and purifying proteins are well known in the art.

5           Alternatively, in one aspect the present invention may be practiced in yeast cells such as *S. cerevisiae* to identify or further characterize protein-protein interactions of the identified intracellular OP-1 modulating factors of the present invention. For example, the yeast two hybrid system, as described in Kalpana et al. (1993) 90 *Proc. Natl. Acad. Sci. U.S.A.* (22): 10593-10597 and already well known to those skilled in the art, is useful for delineating  
10 domains or critical residues for an interaction between two proteins, for example, a protein X and a protein Y. Briefly, hybrid genes are constructed for expression in yeast comprising (a) a DNA binding domain fused to a protein X and (b) an activator domain fused to a protein Y. Transcriptional activation in these experiments is only restored when protein X interacts with protein Y leading to close contact of both the binding domain and the activation domain.  
15 Transcription activation is then monitored by a reporter gene resident in the yeast strain.

#### B. Exemplary Vectors/Vector Construction Considerations

Useful vectors for use in the invention include, but are not limited to plasmids, cosmids, phagemids, yeast artificial chromosomes or other large vectors. Vectors that can be maintained within the nucleus or integrated into the genome by homologous recombination are also useful.

20           Selected portions of non-coding OP-1 sequence can be cloned into a useful vector using standard molecular cloning techniques, as exemplified below. Restriction endonuclease sites will be utilized when possible, and can be engineered into the sequence when needed. Restriction endonuclease sites can be engineered into the non-coding sequence using the common techniques such as site directed mutagenesis and PCR with primers including the  
25 desired restriction endonuclease site.

Also envisioned is a nucleic acid construct comprising a small fragment of 5' non-coding OP-1 sequence in combination with additional conserved elements such as one or more Pax 6 binding sequences and/or Pax 2 binding sequences in operative association with a reporter gene.

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A range of useful 5' non-coding fragments is provided herein, and as will be apparent to those of ordinary skill in the art, smaller fragments of OP-1 sequence also are useful. Such smaller fragments can be identified by deleting bases from one or both ends of the provided 5' non-coding fragments, using techniques that are well known in the art and testing the truncated constructs for their ability to modulate reporter gene expression. In this way, the shortest modulating sequences can be identified.

#### C. Transfection Considerations

Any routine method for incorporating nucleic acids into cells of interest is contemplated in the method of the invention. For example, calcium phosphate ( $\text{CaPO}_4$ ), followed by glycerol shock is a standard means used in the art for introducing vectors, particularly plasmid DNA into mammalian cells. A representative method is disclosed in Cockett et al., (1990) *Biotechnology* 8: 662-667, incorporated herein by reference. Other methods that may be used include electroporation, protoplast fusion (particularly useful in myeloma transfections), microinjections, lipofections and DEAE-dextran mediated uptake. Methods for these procedures are described in F.M. Ausubel, ed., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1989). An important aspect of the invention is the DNA sequences with which the cell is transfected, rather than the mechanical or chemical process by which the DNA incorporation is accomplished.

As will be appreciated by those having skill in the art, optimal DNA concentrations per transfection and other standard conditions will vary according to the transfection protocol. For calcium phosphate transfection, for example, preferably 5-10  $\mu\text{g}$  plasmid DNA per plasmid type is transfected. In addition, the DNA to be transfected preferably is essentially free of contaminants that may interfere with DNA incorporation. A standard means used in the art for purifying DNA is by ethidium bromide banding.

#### D. Exemplary Reporter Genes

Reporter genes are characterized as being easy to transfect into a suitable host cell, easy to detect using an established assay protocol, and genes whose expression can be tightly regulated. Other reporter genes contemplated to have utility include, without limitation, the

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luciferase gene, the Green Fluorescent Protein (GFP) gene, human growth hormone, GAL4 and  $\beta$ -galactosidase.

A well-recognized reporter system is the firefly luciferase reporter system. See, for example Gould, S.J., and Subramani, S. (1988) *Anal. Biochem.*, 17:404-408 for a description of the reporter gene and general methodology. The luciferase assay is fast and has increased sensitivity. Further, the half-life of luciferase protein is short and therefore allows for accurate kinetic studies of luciferase production. The system also is particularly useful in bulk transfections or if the promoter of interest is weak. In this assay transfected cells are grown under standard conditions, and when cultured under assay conditions both ATP and the substrate luciferin is added to the cell lysate. The enzyme luciferase catalyzes a rapid, ATP dependent oxidation of the substrate which then emits light. The total light output is measured using a luminometer according to manufacturer's instructions (e.g., Promega) and is proportional to the amount of luciferase present over a wide range of enzyme concentrations. For example, a vector such as pGL2-LUC (Promega) is particularly useful.

A second well-known reporter system is based on immunologic detection of hGH, it is quick and easy to use. (Selden, R., Burke-Howie, K. Rowe, M.E., Goodman, H.M., and Moore, D.D. (1986), *Mol. Cell. Biol.*, 6:3173-3179 incorporated herein by reference). hGH is assayed in the media, rather than in cell extracts. This allows direct monitoring over by a single population of transfected cells over time.

Additional useful reporter genes are any well characterized genes the expression of which is readily assayed, and examples of such reporter genes can be found in, for example, F.A. Ausubel et al., Eds., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, (1989). As will be appreciated by those having ordinary skill in the art, the listed reporter genes are only a few of the possible reporter genes, and it is only for ease of description that all available reporter genes are not listed. It will be apparent to those of ordinary skill in the art that genes encoding other detectable proteins of interest, such as the gene encoding human OP-1 shown in SEQ. ID No. 1, or other morphogen whose enhanced expression is desirable, is also within the scope of a suitable reporter gene.

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As indicated above and as will be appreciated by those having ordinary skill in the art, particular details of the conventional means for transfection, expression, and assay of recombinant genes are well documented and are understood by those having ordinary skill in the art. The instant invention enables and discloses vectors, cells and a method for screening  
5 compounds to determine the capability of compounds to modulate the expression of OP-1 via the non-coding sequences of the OP-1 genomic DNA.

Further details on the various technical aspects of each of the steps used in recombinant production of foreign genes in mammalian expression systems can be found in a number of texts and laboratory manuals in the art, such as, for example, F.M. Ausubel et al., Ed., *Current*  
10 *Protocols in Molecular Biology*, John Wiley & Sons, New York, (1989).

In view of this disclosure and the examples provided below, a method for identifying molecules which can affect OP-1 expression in a particular cell type *in vivo* now is provided.

Example 1: Cloning of Human OP-1 Gene Non-coding Sequences

Human OP-1 upstream non-coding sequence was obtained by screening the human  
15 genomic library, HL1067J. (Clontech). The library was screened by an initial plating of 750,000 plaques (approximately 50,000 plaques/plate). Hybridizations were done in 40% formamide, 5 x SSPE, 5 x Denhardt's solution, and 0.1% SDS at 37°C using a <sup>32</sup>P-labeled probe made from a human 0.47 kb EcoRI OP-1 cDNA fragment containing mainly 5' non-coding and exon 1 sequences. Nonspecific counts were removed in 0.1 x SSPE, 0.1 % SDS by shaking at 50°C.

20 A 7 kb EcoRI fragment from the human genomic clone, lambda Ö3, was isolated and sequenced and contained 5 kb of OP-1 upstream non-coding sequence.

All sequencing was done according to Sanger et al. (1977) *Proc. Natl. Acad. Sci.* 74:5463-5467, using exonuclease III-mediated unidirectional deletion (Ozkaynak et al., (1987) *BioTechniques*, 5:770-773), subcloning of restriction fragments, and synthetic primers.

25 Compressions were resolved by performing the reactions at 70°C with Taq polymerase and using 7-deaza-GTP (U.S. Biochemical Corp., Cleveland, OH).

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As will be discussed in more detail below, the 1-3317 region of SEQ. ID No. 1 contains a plurality of conserved DNA sequences which share homology with Pax homeobox consensus sequences of Epstein et al. (1994) *J. Biol. Chem.* 269:8355-8361, and have been identified and designated herein as Pax responsive OP-1 modulating elements, comprising approximately  
5 nucleotides 108-121, 139-154, 157-167, 365-378, 491-503, 497-511, 737-747, 891-903, 994-1006, 1123-1140, 1144-1161, 1285-1297, 598-613, 1750-1762, 2001-2023, 2365-2378, 2931-2944 of SEQ. ID No. 1. Particularly preferred Pax 6 responsive OP-1 modulating elements reside at approximately nucleotides 108-121, 139-154, 365-378, 497-511, 598-613, 1750-1762, 2365-2378 and 2931-2944. Other preferred Pax 6 elements include approximately 157-167,  
10 1123-1140, 1144-1161, 1285-1297 and 2001-2023. Particularly preferred Pax 2 responsive OP-1 modulating elements reside at approximately nucleotides 491-503, 737-747, 891-903 and 994-1006.

Still other preferred Pax 6 responsive OP-1 modulating elements reside at approximately 104-120, 105-121, 142-158, 362-378, 497-513, 1750-1766, 2362-2378, 2928-2944, 1128-1144,  
15 1125-1141, 1143-1159, 2000-2016, 2003-20019, 2007-2023, 601-617, 595-611, and 1746-1762. Still other preferred Pax 2 responsive OP-1 modulating elements reside at approximately 493-503, 891-901 and 996-1006.

The transcription initiation site for the human OP-1 gene is at base 2790 of SEQ. ID No. 1. The OP-1 protein translation initiation site is nucleotide 3318 of SEQ. ID No. 1.

20 As described above, osteogenic protein-1 plays a critical role in modulating mesenchymal differentiation and inducing the process of cartilage and bone formation. It is also required for kidney development and is shown to prevent kidney damage from ischemia/reperfusion injury in rats. As presented below in Example 2, functional analysis of the human OP-1 gene promoter has been carried out. The OP-1 promoter and 5' truncated versions of it were placed upstream of  
25 the luciferase coding region which served as the reporter gene. In certain embodiments, the results of the luciferase assays after transient transfection of these constructs into a human kidney cell line, G401, indicate that this upstream sequence has promoter activity. This activity resides about 300 to 700 nucleotides upstream of the first ATG of the OP-1 gene. This promoter activity is constitutive and is not influenced by transactivating agents, e.g., Pax 2 and Pax 6 (see Figure

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1B) which are involved in epithelial/mesenchymal interactions during tissue morphogenesis. In other embodiments drawn to the region between 700 to 3300 nucleotides upstream of the OP-1 gene, however, the data indicate the presence of potential silencer elements of the promoter. Moreover, this region of the promoter can be influenced by a host of factors, including Pax 2, Pax 6 and Retinoic Acid, as illustrated in Example 2.

Both transient and permanent G401 cell lines containing the below-described constructs have been isolated and tested. Other host cell lines including Human Retinoblastoma Y79, Rat Osteosarcoma ROS and Porcine Proximal Tubule LLCPK1 have been tested in transient transfection assays. These data are expected to illustrate similar Pax-mediated regulatory considerations and tissue specificity, thereby permitting even further identification of tissue- and developmental-specific morphogen modulators.

#### Example 2: Analysis of OP-1 Gene Expression

In one aspect, this invention presents a method in which OP-1 non-coding sequences are assayed while in operative association with a reporter gene and modulator compounds such as Pax 2 or Pax 6 or analogs thereof, are thereby tested for their influence on the expression of OP-1. For example, non-coding sequences which are involved in the modulation of OP-1 expression via Pax gene products or functional equivalents or analogs thereof will be identified by (1) transfecting a cell with one or more Pax protein expression vectors and an expression vector comprising OP-1 non-coding sequences in operative association with a reporter gene; (2) culturing the transfectants with one or more candidate compounds; (3) measuring the level of reporter gene expression; and (4) comparing this level of expression to the level of reporter gene expression in the absence of the compound(s). Alternatively, non-coding sequences can be assayed for their responsiveness to compounds which can mimic Pax 2 and/or Pax 6, i.e., Pax 2 and/or Pax 6 analogs. The protocol of the present invention is based on a procedure for identifying compounds which alter, either directly or indirectly, endogenous levels of morphogen expression.

It is also contemplated that candidate compounds may be administered *in vivo* to modulate the level of an endogenous protein, such as OP-1. This can be accomplished by detection of the expression product either at the protein or RNA level.

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Cultured cells are transfected with portions of OP-1 non-coding sequences in operative association with a reporter gene, and such transfected cells are maintained with the vector remaining as a plasmid in the cell nucleus, or the vector can be integrated into the host cell genome, preferably at the OP-1 genomic locus. Cell samples for testing the level of reporter gene expression are collected periodically and evaluated for reporter gene expression using the appropriate assay for the given reporter gene as indicated in the section describing reporter gene assays, or, alternatively, a portion of the cell culture itself can be collected periodically and used to prepare polyA(+) RNA for mRNA analysis. For example, to ascertain the particular time point at which OP-1 is produced following treatment with a candidate morphogen, cells treated with the candidate OP-1 modulating compound, cells treated with compound are collected periodically and evaluated for OP-1 production, as described above. To monitor *de novo* OP-1 synthesis, some cultures are labeled according to conventional procedures with an <sup>35</sup>S-methionine/<sup>35</sup>S-cysteine mixture for 6-24 hours and then evaluated to quantitate OP-1 synthesis by conventional immunoassay methods. Alternatively, anti-OP-1 antibodies can be labeled and incubated with the cells or cell lysates, and the bound complexes detected and quantitated by conventional means, such as those described herein above. Tissues can also be examined directly for the synthesis of OP-1 mRNA using the art-recognized technique of *in situ* hybridization.

Once candidate compounds are identified, they can be produced in reasonable, useful quantities using standard methodologies known in the art. Amino acid-based molecules can be encoded by synthetic nucleic acid molecules, and expressed in a recombinant expression system as described herein above or in the art. Alternatively, such molecules can be chemically synthesized, e.g., by means of an automated peptide synthesizer, for example. Non-amino acid-based molecules can be produced by standard organic chemical synthesis procedures.

Morphogen agonists are anticipated to have utility in any application where tissue morphogenesis is desired, such as in the regeneration of damaged tissue resulting from mechanical or chemical trauma, degenerative diseases, tissue destruction resulting from chronic inflammation, cirrhosis, inflammatory diseases, cancer and the like, and in the regeneration of tissues, organs and limbs. Morphogen antagonists are envisioned to have utility in applications where tissue morphogenesis is to be limited as, for example, in the treatment of malignant

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transformations including, but not limited to, osteosarcomas, Paget's disease, and fibrodysplasia ossificans progressiva (See, for example, Roush (1996) *Science* 273:1170). The ability to detect OP-1 protein in solution provides a valuable tool for diagnostic assays, allowing us to monitor the levels of OP-1 free in the body, e.g., in serum, urine, spinal or peritoneal fluid, breast exudate, and other body fluids in order to assess the presence of OP-1 modulating activity. The present invention therefor provides a means of identifying naturally occurring or synthetic proteins or other factors or drugs that have OP-1 modulating activities. Moreover, compounds that induce the expression of Pax 2 or Pax 6 are also particularly useful.

OP-1 is expressed in a variety of different cell types, including renal, bone, lung, heart, uterine, cardiac and neural tissue. Candidate compounds can be identified which have a modulating effect on cells of one tissue type but not another, and/or wherein the effect is modulated differently in the different cells. The assay described below can be used to evaluate the effect of a candidate compound in a particular cell type known to express OP-1 under physiological conditions.

The present invention therefor also provides a test cell or cell line which expresses an exogenously introduced OP-1 protein, which is responsive to morphogen modulators such as Pax 2 and/or Pax 6 or analogs thereof as contemplated herein. Said cell or cell line can be also used as described herein, to replace cell lines that produce OP-1 endogenously, for the screening assays described herein above. Further, the invention provides a test cell that is transfected with DNA encoding Pax gene products (e.g., Pax 2 and/or Pax 6) for the modulation of endogenous or exogenous OP-1 gene expression.

While a readily assayable, well characterized, non OP-1 reporter gene is preferred in the screening method disclosed herein, as will be appreciated by those having ordinary skill in the art, OP-1 coding sequence also may be used in the method of the invention providing an improved method for the production of high levels of OP-1, exploiting the high level of induction of gene expression of the present invention. The OP-1 expression preferably is determined by an immunoassay or by Northern or dot blot or other means for measuring mRNA transcript. See, for example, WO 95/11983, published May 4, 1995 for a detailed description on assaying changes in OP-1 levels in a cell or fluid. Further, the cells of the present invention can

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be used in the analysis of the transcription factors, for example, DNA-protein and/or protein-protein interactions involved in OP-1 transcription regulation or that of other morphogens.

Provided below is an exemplary protocol for carrying out the method of the invention, using the luciferase gene as the reporter gene and a mammalian cell line known to express OP-1.

5 The example is non-limiting, and other cells, reporter genes and morphogen non-coding sequences such as, but not limited to, an OP-1 non-coding sequence, are envisioned.

The OP-1 genomic DNA nucleotides 1-3317 of SEQ. ID No. 1 was used to prepare a series of deletion constructs carrying the luciferase reporter gene and portions of the OP-1 gene non-coding region (Figure 1A). The pGL2-Basic plasmid comprising a nucleotide sequence  
10 encoding the detectable enzyme luciferase (Promega, Madison WI) was employed as the basic vector. The OP-1 promoter sequence (corresponding to nucleotides 1 to 3305 of SEQ. ID No. 1) was then inserted into the pGL2-Basic plasmid (construct pAS3.3, Figure 1A). Nucleotide +1 is 30 nucleotides downstream of the Hind III site present in the OP-1 genomic sequence. There are therefore an additional 30 base pairs of DNA sequence upstream of position +1 in the OP-1  
15 promoter, comprising AAGCTTGATG CCTGCACAGT CAGCCCTCAG, wherein AAGCTT corresponds to a Hind III site.

The genomic sequence was digested with Hind III and BamHI. The BamHI site was introduced into the OP-1 promoter at nucleotides 3306 to 3311 of SEQ. ID No. 1 by site-directed mutagenesis according to methods well known in the art using the following reverse complement  
20 primer: 5' CAT CGC GCC GGA TCC ACG CGC TAC CCG GGC 3' wherein GGATCC corresponds to a BamHI site.

Thus the mutagenesis generated the following change in the OP-1 sequence:

native seq: 5' GCC CGG GTA GCG CGT AGA GCC GGC GCG ATG 3'

altered seq: 5' GCC CGG GTA GCG CGT GGA TCC GGC GCG ATG 3'

25 This resulted in the following changes in the OP-1 promoter in construct pAS3.3:  
GCCCGGGTAG CGCGTGGATC TAAGTAAGCT TGGCATTCCG GTACTGTTGG

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TAAAATG wherein the G represents nucleotide 3306 of SEQ. ID No. 1 and wherein ATG corresponds to the luciferase ATG (nucleotides 3318 to 3320 of SEQ. ID No. 1).

Therefore, the native OP-1 promoter sequences stop at nucleotide 3305, nucleotide 3306 results from an A to G nucleotide change, and nucleotides 3307 to 3345 are derived from the pGL2-Basic vector. Thus, 3335 nucleotides (30 nucleotides upstream of nucleotide 1 up to 3305 of SEQ. ID No. 1) of cloned 5' non-coding sequence of the OP-1 gene was placed in transcriptionally operative association with the luciferase reporter gene.

Serial 5' deletions (Figure 1A) were constructed by digestion of the pAS3.3 construct with a series of restriction enzymes followed by religation of the plasmid according to well-known cloning techniques. For example, the pAS3.3 vector was cleaved with BglII (present in the pGL2-Basic vector and in the OP-1 promoter) and recircularized to obtain pAS2.5; the pAS3.3 vector was cleaved with KpnI (present in the pGL2-Basic vector and in the OP-1 promoter) and recircularized to obtain pAS1.2; the pAS3.3 vector was cleaved with XhoI (present in the pGL2-Basic vector) and SacII, followed by a T4 DNA polymerase fill-in reaction and recircularized to obtain pAS0.8; the pAS3.3 vector was cleaved with KpnI (present in the pGL2-Basic vector) and AatII followed by a T4 DNA polymerase fill-in reaction and recircularized to obtain pAS0.7; the pAS3.3 vector was cleaved with XhoI (present in the pGL2-Basic vector) and PvuII followed by a T4 DNA polymerase fill-in reaction and recircularized to obtain pAS0.6; the pAS3.3 vector was cleaved with XhoI (present in the pGL2-Basic vector) and AvrII followed by a T4 DNA polymerase fill-in reaction and recircularized to obtain pAS0.3; and the pAS3.3 vector was cleaved with KpnI (present in the pGL2-Basic vector) and PstI followed by a T4 DNA polymerase fill-in reaction and recircularized to obtain pAS0.1.

The vectors were transfected into G401 human kidney cells, either alone or together with the cDNA for human Pax 2 and/or for human Pax 6, previously cloned into a pCMV $\beta$  expression vector (Clontech), with the removal of the  $\beta$ -galactosidase gene by standard methods (Epstein, et al. (1994) *J. Biol. Chem.* 269:8355-8361), the disclosure of which is incorporated herein by reference. Briefly, cells were plated in 60 mm petri dishes ( $5 \times 10^5$  cell/dish) in McCoy's 5A medium containing 10% fetal bovine serum. Twenty-four hours later, the above-described vectors were transfected into the cultured cells, using a Lipofectamine (BRL/Gibco) method in

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serum-free medium (Optimem, BRL) for 6 hours. Cells were transfected with (a) an OP-1 promoter constructs alone; (b) an OP-1 promoter construct + the Pax 2 construct; (c) an OP-1 promoter construct + the Pax 6 construct; or (d) an OP-1 promoter construct + the Pax 2 construct + the Pax 6 construct. Control transfected cells contained the pGL-Basic plasmid without OP-1 non-coding sequences as a negative control and pSV-Luc, which has an SV40 promoter, as a positive control. Thereafter, transfected cells were cultured in complete media. Seventy-two hours later, luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison WI) and a luminometer (DynaTech). The results are displayed in Figure 1B and discussed below.

The luciferase production by cells containing the pAS3.3 construct, comprising 3305 nucleotides upstream of the translation start site of the OP-1 gene, was induced 2.4 fold by co-transfection with the Pax 2 cDNA and 9.6 fold by co-transfection with the Pax 6 cDNA (Figure 1B). The triple transfection of both the Pax 2 and Pax 6 expression vectors with the pAS3.3 construct resulted in an additive 11.5 fold induction suggesting that Pax 2 and Pax 6 gene products have an additive effect on OP-1 gene expression. Cells containing the 5' deletion mutants of the pAS3.3 construct were less responsive to the inductive effect of co-transfection with Pax 2 or Pax 6 constructs. For example, the pAS2.5 construct was only induced 1.7 fold by co-transfection with the Pax 2 construct and this Pax 2 responsiveness was abolished by further deletion of about 1.3kb of 5' sequence, with no Pax 2 induction detected for the pAS1.2 construct or the remaining constructs with further 5' deletions.

Pax 6 responsiveness was also lost by about 42% upon deletion of 793 bases from the pAS3.3 construct. A 4 to 5.5 fold induction of luciferase production was demonstrated in the cells comprising the Pax 6 construct co-transfected with pAS2.5 (+794 to +3305), pAS1.2 (+2068 to +3305), pAS0.8 (+2501 to +3305), and pAS0.7 (+2606 to +3305) of SEQ. ID No. 1 but this Pax 6 responsiveness was lost upon deletion of nucleotides +2606 to +2690. This suggests that this 85 nucleotide region contains preferred sequences necessary for OP-1 modulation by Pax gene products or analogs thereof.

Constructs pAS3.3 and pAS2.5, comprising 3.3kb 5' non-coding region (1 to 3305 of SEQ. ID No. 1) and a 793 nucleotide 5' deletion thereof, respectively, produced less luciferase

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constitutively than the other deletion mutants, suggesting the presence of negative regulatory elements in the 5' non-coding region comprising nucleotides 1 to 2067 (Figure 1B).

The complete DNA sequence of the 5' non-coding upstream OP-1 gene (1 to 3317 of SEQ. ID No. 1) was analyzed using a string search program to identify putative Pax 2 or Pax 6 binding motifs, relying in part on the Pax 2 and Pax 6 consensus sequences described by Epstein et al. (1994) *J. Biol. Chem.* 269:8355-8361, and in part on certain other suitable Pax consensus sequences disclosed herein. One consensus sequence for Pax 6 is T T C A C G C A/T T G/C A N T G/T A/C N T/C corresponding to SEQ. ID No. 2 wherein the N is either A, G, C or T. One consensus sequence for Pax 2 is G T C A C/T G C G/A T G A and corresponds to SEQ. ID No. 3. The string search program allowed for partial mismatches and revealed a number of Pax 6 or Pax 2 responsive regions. Currently preferred Pax 2 responsive regions are located at approximately 491-503, 737-747, 891-903, 994-1006 of SEQ. ID No. 1 and share approximately 69-82% identity with the Pax 2 consensus sequence of SEQ. ID. No. 3.

Currently preferred Pax 6 responsive elements include approximately nucleotides 108-121, 139-154, 365-378, 497-511, 1750-1762, 2365-2378, 2931-2944 of SEQ. ID No. 1. These Pax 6 responsive elements share between 53% and 82% identity with the Pax 6 consensus sequence of SEQ. ID No. 3. Other currently preferred Pax 6 responsive elements include approximately nucleotides 157-167, 1123-1140, 1144-1161, 1285-1297, and 2001-2023 of SEQ. ID No. 1.

It is understood that some of the consensus Pax motifs may function to regulate OP-1 gene expression, where others may not. Further, characterization of each putative Pax responsive OP-1 element is accomplished as described below in Example 5. As earlier discussed, it is contemplated that Pax responsive elements, such as those described herein, are present in the promoter sequences of other morphogens as defined herein and thereby can be identified and exploited as taught herein. Pax responsive elements, as contemplated by the present invention, modulate the *in vivo*, *ex vivo* and *in vitro* expression of morphogens and thus are useful for identifying particular compounds that can modulate particular morphogens.

It is contemplated that the present invention further provides a useful method for identifying nucleic acids and their respective encoded compounds capable of modulating Pax

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mediated OP-1 gene regulation. In one embodiment, one or more candidate nucleic acids which encode a candidate compound or compounds capable of intracellular modulation of OP-1 gene expression, mediated directly or indirectly by Pax responsive elements located in the OP-1 gene 5' non-coding region (nucleotides 1-3317 of SEQ. ID No. 1), are also transfected into the cells of the invention comprising Pax responsive OP-1 5' non-coding sequence/reporter gene vectors. "Candidate nucleic acid" or "candidate DNA" or "candidate RNA" as defined herein, is therefore a nucleic acid (e.g. a DNA or RNA) that encodes a compound that modulates OP-1 gene expression via a Pax responsive element. In one embodiment, a candidate DNA can encode a transcription factor that shares functional similarity with a Pax gene product or is a Pax analog or agonist. Alternatively, the candidate nucleic acid can encode a compound that behaves as an inhibitor of OP-1 gene expression or is a Pax antagonist. In another embodiment, a candidate RNA or RNAs may be first isolated from a cell or synthesized *in vitro* by methods well known to the skilled artisan and then used to transfect the above-described cell. It is also, contemplated that tissue specific candidate nucleic acids can be identified and isolated according to the method of the present invention. Thus, for example, expression vectors comprising tissue specific candidate DNAs can be co-transfected into a cell together with an expression vector containing the OP-1 5' non-coding sequence of nucleotides 1-3317 of SEQ ID No 1, or truncated versions thereof, in operative association with a luciferase reporter gene. Alterations in luciferase activity relative to control transfections is an indication that the candidate DNA encodes a compound capable of modulating OP-1 gene regulation. It is contemplated that individual nucleic acids or pools of nucleic acids, for example, a cDNA library or a pool of synthetic RNAs, may be screened for their ability to modulate OP-1 gene expression via the responsive elements of the present invention in accordance with the methods of the invention.

### Example 3. Further Characterization of the Pax Responsive OP-1 Modulating Elements

To characterize the respective roles of certain of the Pax 2 (for example, approximately, nucleotides 491-503, 737-747, 891-903, 994-1006 of SEQ. ID No. 1) and Pax 6 responsive elements (for example, approximately, nucleotides 108-121, 139-154, 157-167, 365-378, 498-511, 598-613, 1123-1140, 1144-1161, 1285-1297, 1750-1762, 2001-2023, 2365-2378, 2931-2944 of SEQ. ID No. 1) in the modulation of the OP-1 gene expression, mutations of the various Pax 2 and Pax 6 responsive elements are analyzed. A loss or decrease of Pax mediated OP-1

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gene expression by mutation of a portion of each Pax 2 responsive element sequence indicates a role for that sequence in Pax mediated OP-1 gene regulation. Conversely, an increase in Pax mediated OP-1 gene expression of the mutated Pax responsive elements may indicate, for example, enhanced binding of Pax transcription factors to the altered DNA recognition sequence or, alternatively, a loss or decrease in binding of one or more negative regulatory elements.

Further, the OP-1 gene promoter is analyzed by DNase footprinting to further define the Pax responsive regions and to determine binding specificity and affinity of the Pax gene products required for the expression of the OP-1 gene. DNase footprinting is carried out according to techniques well known to those skilled in the art.

In addition, the interaction of the OP-1 promoter with nuclear proteins is further characterized by gel shift assays using oligonucleotide probes corresponding to the Pax 2 consensus sequence (SEQ. ID No. 3) or the Pax 6 consensus sequence (SEQ. ID No. 2) or variants thereof, with and without mutations in the oligonucleotide sequences. Briefly, cells are lysed according to art known methods and nuclear extracts are prepared and incubated with a radiolabelled oligonucleotide of choice, by methods well known to the skilled artisan. Binding of one or more of the protein components of the extract to the oligonucleotide produces DNA/protein complexes having retarded electrophoretic mobility relative to the mobility of the uncomplexed oligonucleotide DNA probe. Completion of radiolabelled oligonucleotide binding with cold (unlabelled) oligonucleotide is performed to confirm the specificity of DNA/protein complexes. These assays can demonstrate alterations in the amount or activity of a nuclear extract component in response to treatment with candidate compounds.

Supershift assays, in which the nuclear extract oligonucleotide complexes are further retarded by complexing with specific antibody, are used to determine the identity of the Pax gene product or analog thereof. In these assays, the previously described protein-oligonucleotide probe complexes are further incubated with specific antibody and subjected to electrophoresis, according to art known methods. The DNA-protein complexes from a test cell induced, for example, by a candidate compound, are supershifted during gel analysis when treated with an anti-Pax 2 or anti-Pax 6 antibody, or other Pax family antibodies, for example. This supershift can be reversed by incubation with the antigenic peptide.

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The tissue specificity of the Pax-DNA complexes is further defined by a comparison of gel shift and supershift mobilities using cell extracts from various tissues and cells. These studies may reveal the involvement of one or more tissue specific factors that may participate in Pax mediated regulation of OP-1 gene transcription.

5    Example 4: *In vivo* Animal Model for Testing Efficacy of Compounds to Modulate OP-1 Expression

It previously has been demonstrated that OP-1 can be an effective treatment for osteoporosis on the standard ovariectomized rat model, as indicated by the dose-response increase in alkaline phosphatase and osteocalcin levels following injection with OP-1. The  
10    osteoporotic rat model provides an *in vivo* model for evaluating the efficacy of a candidate compound for modulating morphogen synthesis. In order to determine the effect of a candidate morphogen stimulating agent on OP-1 production and, thereby, on bone production *in vivo*, alkaline phosphatase and osteocalcin levels are measured under conditions which promote osteoporosis, e.g., wherein osteoporosis is induced by ovary removal in rats and in the presence  
15    and absence of a candidate compound. A compound competent to enhance or induce endogenous OP-1 expression should result in increased osteocalcin and alkaline phosphatase levels.

Forty Long-Evans rats (Charles River Laboratories, Wilmington) weighing about 200g each are ovariectomized (OVX) using standard surgical procedures, and ten rats are sham  
20    operated. The ovariectomization of the rats produces an osteoporotic condition within the rats as a result of decreased estrogen production. Food and water are provided *ad libitum*. Eight days after ovariectomy, the rats, prepared as described above, are divided into three groups: (a) sham-operated rats; (b) ovariectomized rats receiving 1 ml of phosphate-buffered saline (PBS) i.v. in the tail vein; and (c) ovariectomized rats receiving various dose ranges of the candidate  
25    morphogen stimulating agent either by intravenous injection through the tail vein or direct administration to kidney tissue.

The effect of the candidate compound on *in vivo* bone formation can be determined by preparing sections of bone tissue from the ovariectomized rats. Each rat is injected with 5 mg of tetracycline, which will stain the new bone (visualized as a yellow color by fluorescence), on the

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15th and 21st day of the study, and on day 22 the rats are sacrificed. The body weights, uterine weights, serum alkaline phosphatase levels, serum calcium levels and serum osteocalcin levels then were determined for each rat. Bone sections are prepared and the distance separating each tetracycline staining is measured to determine the amount of new bone growth. The levels of  
5 OP-1 in serum following injection of the candidate agent also can be monitored on a periodic basis using routine methods.

#### Example 5: Determination of OP-1 Protein Production

Where OP-1 acts as the reporter gene, detection of the gene product readily can be assayed using antibodies specific to the protein and standard immunoassay testings. For  
10 example, OP-1 may be detected using a polyclonal antibody specific for OP-1 in an ELISA, as follows.

1  $\mu$ g/100  $\mu$ l of affinity-purified polyclonal rabbit IgG specific for OP-1 is added to each well of a 96-well plate and incubated at 37°C for an hour. The wells are washed four times with 0.167M sodium borate buffer with 0.15 M NaCl (BSB), pH 8.2, containing 0.1% Tween 20. To  
15 minimize non-specific binding, the wells are blocked by filling completely with 1% bovine serum albumin (BSA) in BSB and incubating for 1 hour at 37°C. The wells are then washed four times with BSB containing 0.1% Tween 20. A 100  $\mu$ l aliquot of an appropriate dilution of each of the test samples of cell culture supernatant is added to each well in triplicate and incubated at 37°C for 30 min. After incubation, 100  $\mu$ l biotinylated rabbit anti-OP-1 serum (stock solution is  
20 about 1 mg/ml and diluted 1:400 in BSB containing 1% BSA before use) is added to each well and incubated at 37°C for 30 min. The wells are then washed four times with BSB containing 0.1% Tween 20. 100  $\mu$ l streptavidin-alkaline (Southern Biotechnology Associates, Inc. Birmingham, Alabama, diluted 1:2000 in BSB containing 0.1% Tween 20 before use) is added to each well and incubated at 37°C for 30 min. The plates are washed four times with 0.5M Tris  
25 buffered Saline (TBS), pH 7.2. 50  $\mu$ l substrate (ELISA Amplification System Kit, Life Technologies, Inc., Bethesda, MD) is added to each well incubated at room temperature for 15 min. Then, 50  $\mu$ l amplifier (from the same amplification system kit) is added and incubated for another 15 min. at room temperature. The reaction is stopped by the addition of 50  $\mu$ l

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0.3 M sulfuric acid. The OD at 490 nm of the solution in each well is recorded. To quantitate OP-1 in culture media, a OP-1 standard curve is performed in parallel with the test samples.

Example 6: Production of OP-1 Polyclonal and Monoclonal Antibodies

5 Polyclonal antibody for OP-1 protein may be prepared as follows. Each rabbit is given a primary immunization of 100 µg/500 µl *E. coli* produced OP-1 monomer (amino acids 328-431 in SEQ ID No:5) in 0.1% SDS mixed with 500 µl Complete Freund's Adjuvant. The antigen is injected subcutaneously at multiple sites on the back and flanks of the animal. The rabbit is boosted after a month in the same manner using incomplete Freund's Adjuvant. Test bleeds are taken from the ear vein seven days later. Two additional boosts and test bleeds are performed at  
10 monthly intervals until antibody against OP-1 is detected in the serum using an ELISA assay. Then, the rabbit is boosted monthly with 100 µg of antigen and bled (15 ml per bleed) at days seven and ten after boosting.

Monoclonal antibody specific for OP-1 protein may be prepared as follows. A mouse is given two injections of *E. coli* produced OP-1 monomer. The first injection contains 100µg of  
15 OP-1 in complete Freund's adjuvant and is given subcutaneously. The second injection contains 50 µg of OP-1 in incomplete adjuvant and is given intraperitoneally. The mouse then receives a total of 230 µg of OP-1 in four intraperitoneal injections at various times over an eight month period. One week prior to fusion, both mice are boosted intraperitoneally with 100 µg of OP-1 and 30 µg of the N-terminal peptide (Ser<sub>293</sub>-Asn<sub>309</sub>-Cys) conjugated through the added cysteine  
20 to bovine serum albumin with SMCC crosslinking agent. This boost was repeated five days (IP), four days (IP), three days (IP) and one day (IV) prior to fusion. The mouse spleen cells are then fused to myeloma cells at a ratio of 1:1 using PEG 1500 (Boeringer Mannheim), and the cell fusion is plated and screened for OP-1-specific antibodies using OP-1 as antigen. The cell fusion and monoclonal screening are then performed according to standard procedures widely available  
25 in the art.

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Example 7: Process for Detecting OP-1 in Serum

Presented below is a sample protocol for identifying OP-1 in serum. Following this general methodology, OP-1 may be detected in body fluids, including serum, and can be used in a protocol for evaluating the efficacy of an OP-1 modulating compound *in vivo*.

5 A monoclonal antibody raised against mammalian, recombinantly produced OP-1 using standard immunology techniques well described in the art and described generally in example 6, above, was immobilized by passing the antibody over an agarose-activated gel (e.g., Affi-Gel™, from Bio-Rad Laboratories, Richmond, CA, prepared following manufacturer's instructions) and used to purify OP-1 from serum. Human serum then was passed over the column and eluted  
10 with 3M K-thiocyanate. K-thiocyanate fractions then were dialyzed in 6M urea, 20mM PO<sub>4</sub>, pH 7.0, applied to a C8 HPLC column, and eluted with a 20 minute, 25-50% acetonitrile/0.1% TFA gradient. Mature, recombinantly produced OP-1 homodimers elute between 20-22 minutes, and are used as a positive control. Fractions then were collected and tested for the presence of OP-1 by standard immunoblot using an OP-1 specific antibody. Using this method OP-1 readily  
15 was detected in human serum. See also, PCT/US92/07432 for a detailed description of the assay.

Example 8: Considerations for Formulations and Methods for Administering Therapeutic Agents

Where the OP-1-modulating agent identified herein comprises part of a tissue or organ preservation solution, any commercially available preservation solution may be used. For  
20 example, useful solutions known in the art include Collins solution, Wisconsin solution, Belzer solution, Eurocollins solution and lactated Ringer's solution. Generally, an organ preservation solution usually possesses one or more of the following properties: (a) an osmotic pressure substantially equal to that of the inside of a mammalian cell, (solutions typically are  
hyperosmolar and have K<sup>+</sup> and/or Mg<sup>++</sup> ions present in an amount sufficient to produce an  
25 osmotic pressure slightly higher than the inside of a mammalian cell); (b) the solution typically is capable of maintaining substantially normal ATP levels in the cells; and (c) the solution usually allows optimum maintenance of glucose metabolism in the cells. Organ preservation solutions also may contain anticoagulants, energy sources such as glucose, fructose and other sugars, metabolites, heavy metal chelators, glycerol and other materials of high viscosity to enhance

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survival at low temperatures, free oxygen radical inhibiting agents and a pH indicator. A detailed description of preservation solutions and useful components may be found, for example, in US Patent No. 5,002,965.

Where the OP-1-modulating agent is to be provided to an individual, e.g., the donor prior to harvest, or the recipient prior to or concomitant with transplantation, the therapeutic agent may be provided by any suitable means, preferably directly (e.g., locally, as by injection to the tissue or organ locus) or systemically (e.g., parenterally or orally).

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in *Remington's Pharmaceutical Sciences* (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity to help maintain the agent at the desired locus. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, tricalcium phosphate, polybutyrate, lactide and glycolide polymers and lactide/glycolide copolymers, may be useful excipients to control the release of the agent *in vivo*.

As will be appreciated by those skilled in the art, the concentration of the compounds described in a therapeutic composition will vary depending upon a number of factors, including the dosage of the drug to be administered, the chemical characteristics (e.g., hydrophobicity) of the compounds employed, and the route of administration. Where the morphogen-stimulating agent is part of a preservation solution, the dosage likely will depend for example, on the size of the tissue or organ to be transplanted, the overall health status of the organ or tissue itself, the length of time between harvest and transplantation (e.g., the duration in storage), the frequency with which the preservation solution is changed, and the type of storage anticipated, e.g., low temperature. In general terms, preferred ranges include a concentration range between about 0.1 ng to 100 µg/kg per tissue or organ weight per day.

Where the therapeutic agent is to be administered to a donor or recipient, the preferred dosage of drug to be administered also is likely to depend on such variables as the type and extent of progression of the disease, the overall health status of the particular patient, the relative

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biological efficacy of the compound selected, the formulation of the compound excipients, and its route of administration. In general terms, a suitable compound of this invention may be provided in an aqueous physiological buffer solution containing about 0.001% to 10% w/v compound for parenteral administration. Typical dose ranges are from about 10 ng/kg to about 1 g/kg of body weight per day; and preferred dose range is from about 0.1 µg/kg to 100 mg/kg of body weight per day.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Example 9: An AatII-PvuII fragment of the OP-1 upstream region contains an expression silencer

Luciferase levels were assayed in G401 cells transfected with the OP-1 upstream deletion constructs described in Example 2. The G401 cells were transfected with the deletion constructs using Lipofectamine. Forty-eight to seventy-two hours later, luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison WI) and a luminometer (DynaTech). The relative luciferase activities are shown in Figure 2. Maximal activity was observed for the construct containing 0.6 kb of OP-1 upstream DNA. The addition of approximately 0.1 kb of upstream DNA (the AatII-PvuII fragment, approximately nucleotides 2606-2690 of SEQ ID NO: 1) reduces luciferase activity approximately five fold as shown in Figure 2 by the AatII construct containing approximately 0.7 kb of OP-1 upstream DNA.

The expression silencing property of the AatII-PvuII fragment (nucleotides 2606-2690 of SEQ ID NO: 1) was further tested by comparing luciferase expression in pAS3.3 containing cells with luciferase expression in pAS3.3d containing cells. The pAS3.3 construct contains 3.3 kb of OP-1 upstream DNA fused to the luciferase gene. The pAS3.3d construct lacks the AatII-PvuII fragment, and was made by digesting the pAS3.3 construct with AatII and PvuII, blunting the AatII overhang, and religating the vector.

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G401 cells were plated in triplicate in 6 well plates. After a one day incubation, the cultured cells were transfected with the deletion constructs, using Lipofectamine and approximately 1 µg of either pAS3.3 or pAS3.3d. Forty-eight to seventy-two hours later, luciferase activity was measured as described previously. The results are shown in Figure 3.

- 5 Cells containing pAS3.3 expressed approximately three-fold (between two and four-fold) more luciferase than cells containing pAS3.3d. The AatII-PvuII fragment (nucleotides 2606-2690 of SEQ ID NO: 1) therefore contains a silencer of gene expression.

- 10 The luciferase production by cells containing the pAS3.3 construct, comprising 3305 nucleotides upstream of the translation start site of the OP-1 gene, was induced 2.4 fold by co-transfection with the Pax 2 cDNA and 9.6 fold by co-transfection with the Pax 6 cDNA (Figure 1B). The triple transfection of both the Pax 2 and Pax 6 expression vectors with the pAS3.3 construct resulted in an additive 11.5 fold induction suggesting that Pax 2 and Pax 6 gene products have an additive effect on OP-1 gene expression. Cells containing the 5' deletion
- 15 mutants of the pAS3.3 construct were less responsive to the inductive effect of co-transfection with Pax 2 or Pax 6 constructs. For example, the pAS2.5 construct was only induced 1.7 fold by co-transfection with the Pax 2 construct and this Pax 2 responsiveness was abolished by further deletion of about 1.3kb of 5' sequence, with no Pax 2 induction detected for the pAS1.2 construct or the remaining constructs with further 5' deletions.

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## SEQUENCE LISTING

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(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

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(C) CLASSIFICATION:

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17415 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

|                                                                   |     |
|-------------------------------------------------------------------|-----|
| TCAACCGGTC TCTTTAGGTT TTGGCTGTGC TTATTACTAT TCATTCAACA GGTACTAATT | 60  |
| GAGCACCTGC TGTGTGCCAG GCTCAGAATA GGCTCAGGTG AGATGCACAA AGAAGGGTAA | 120 |
| ACTAGAATCC TTGCTTAGAC ACTGACGGAT CAGTTGTTTC ATATGTAAAT TGTAGCACCA | 180 |
| AGACCTGCTG CCCCTGCCCC CAGCCTCACC TGCTTGTGAA GATCCCTCCA AAAGATTGTA | 240 |

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|    |            |            |             |             |            |            |      |
|----|------------|------------|-------------|-------------|------------|------------|------|
|    | GAGTAGATAA | AAAGCAGAGA | CTACTACTGA  | AGAACAGGGC  | TGCTTTGGCT | CCTTATTATT | 300  |
|    | TCAGACTTTG | GAAGAAAATG | ACCTCCTTTT  | TCTCTACTGG  | CACTGAGTGC | ATAGCTGACC | 360  |
| 5  | TAGCAAGCCA | GGCCTGGAGG | GCGTGTGCAG  | GGCTGGGGAC  | CGAGCCTGGT | TTCTGTTCCC | 420  |
|    | TGCTCTGCAG | CTCAAGCACT | TGCTGTTTCCT | CCACCTGGGA  | TGCCTTTCCC | TGGAAAAGCC | 480  |
| 10 | TGTCTCTTTC | TTGTCTTTCA | GGACTCAGGT  | CAGTGGCATC  | TCCTCCAAAA | ACTCCCCCTC | 540  |
|    | CCACCCTCCA | TCACCTCACC | CTGTTTATCT  | GCGCCCCCGC  | CCCCACTGCC | TGTCACTTAT | 600  |
|    | TGCAGGCTGA | AGTGACCCAG | GCTCTCCAGT  | TGTACACTCT  | CAGATGGACC | CTGGACGACT | 660  |
| 15 | GTGGCACTCC | TGCAATTTCC | CCAGTCTCCC  | TGGGGTAGGA  | TTCTTGCTTG | CCAGGATGCC | 720  |
|    | CACCTTTCCT | TCTCCCTCCT | GCATGTCTCT  | CTCTGCCTGG  | CTTCTGAATT | GTTTCCAGAG | 780  |
| 20 | AGAGTGATAG | ACAAGATCTG | CCTCTCCTTC  | AGTCCCTGAA  | TCTTATTTAA | GGCTCTTGCT | 840  |
|    | TTGCTTCCCT | GGCCTGGAGG | CGGCTCCTTG  | ATGGAGTCTG  | CCATGTGGGT | TCGCTCATGG | 900  |
|    | CCATGTCTTC | CTGCCCAGCA | TGGTGCTTGG  | CCCTGGGACT  | GGCCACATAA | TATCTGGGCC | 960  |
| 25 | AGGTGCAAAA | TTAGTACGGG | GCAGGGGGTA  | CTTTGTTTCAT | AGGTGATTCA | GAACCACATA | 1020 |
|    | TGGTGACCTC | AGAGTAGGAA | ACCAAGTGTG  | GGGCCCTTAA  | GAGCTGGGGG | GCCCTGTACG | 1080 |
| 30 | ACTGTCCAGG | TTGCAGGCCC | CACAGCTCGC  | CTCCTGATAT  | CCTGTGCTCC | ATGCTTGTCT | 1140 |
|    | GTTGAAGGAA | GGAGTGAATG | GATGAAGAGC  | AGGTGGTGGG  | GGGTGGTTTG | AGGGCCTTGC | 1200 |
|    | TGGTGGGTGG | GTAGAGGCCC | CTCCCTGGCA  | TGGGGCTCAA  | GACCTGTTCC | ATCCACAGC  | 1260 |
| 35 | CTGGGGCTGT | GTGTAAATGG | CCAGGACCTG  | CAGGCTGGCA  | TTTTTCTGCT | CCTTGCCTGG | 1320 |
|    | CTCTGGCTCC | CCTTTCTCCA | CCCATGTGGC  | CCCTCAGGCT  | GCCATCTAGT | CCAAAAGTCC | 1380 |
| 40 | CAAGGGAGAC | CCAGAGGCCA | CTTGGCAAAC  | TACTTCTGCT  | CCAGAAAACT | GTAGAAGACC | 1440 |
|    | ATAATTCTCT | TCCCCAGCTC | TCCTGCTCCA  | GGAAGGACAG  | CCCCAAAGTG | AGGCTTAGCA | 1500 |
|    | GAGCCCCCTC | CAGACAAGCG | CCCCCGCTTC  | CCCAACCTCA  | GCCCTTCCCA | GTTTCATCCA | 1560 |
| 45 | AAGGCCCTCT | GGGGACCCAC | TCTCTCACCC  | AGCCCCAGGA  | GGGAAGGAGA | CAGGATGAAC | 1620 |
|    | TTTTACCCCG | CTGCCCTCAC | TGCCACTCTG  | GGTGCAGTAA  | TTCCCTTGAG | ATCCACACC  | 1680 |
| 50 | GGCAGAGGGA | CCGGTGGGTT | CTGAGTGGTC  | TGGGGACTCC  | CTGTGACAGC | GTGCATGGCT | 1740 |
|    | CGGTATTGAT | TGAGGGATGA | ATGGATGAGG  | AGAGACAGGA  | GAGGAGGCCG | ATGGGGAGGT | 1800 |
|    | CTCAGGCACA | GACCCCTTGA | GGGGAAGAGG  | ATGTGAAGAC  | CAGCGGCTGG | CTCCCCAGGC | 1860 |
| 55 | ACTGCCACGA | GGAGGGCTGA | TGGGAAGCCC  | TAGTGGTGGG  | GCTGGGGTGT | CTGGTCTCAG | 1920 |
|    | GCTGAGGGGT | GGCTGGAAAG | ATACAGGGCC  | CCGAAGAGGA  | GGAGGTGGGA | AGAACCCCCC | 1980 |
| 60 | CAGCTCACAC | GCAGTTCACT | TATTCACCTA  | ACAAATCGTG  | ACTGCGCACG | TACAGTGGCT | 2040 |
|    | ACCAGGCGCT | GGGTTCAAGG | CACTGCGGGT  | ACCAGAGGTG  | CGGAGAAGAT | CGCTGATCCG | 2100 |
|    | GGCCCCAGTG | CTCTGGGTGT | CTAGCGGGGG  | TAAGAAGGCA  | ATAAAGAAGG | CACGGAGTAA | 2160 |
| 65 | CTCAAACAGC | AATTCCAGAC | AGCAAGAGAA  | ACTACAGGAA  | AGAAAACAAA | CGTGCGAGGG | 2220 |

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|    |            |             |            |            |            |             |      |
|----|------------|-------------|------------|------------|------------|-------------|------|
|    | GCGAGGCGAG | GAAACAACCT  | CAGCTTGGCA | GGTCTTGGAG | GTCTCTGGGA | GGAGAAAGCA  | 2280 |
|    | GCGTCTGATG | GGGGCGGGAG  | GTGGTGAGTG | GGGAGAGGTC | CAGGCGGAGG | GAATGGCGAG  | 2340 |
| 5  | CGAGAGACAG | GCTGGCAACG  | GCTTCAGGGA | GGCGCGGAGG | GGTCAGCGTG | GCTGGCTTAA  | 2400 |
|    | AAGGATACAT | GGGACTAGGG  | GCAAGACCGG | CTCAAGGTCA | CCGCTTCCAG | GACCTTCTAT  | 2460 |
| 10 | TTCCGCGCCA | CCTCCGCGCT  | CCCCAACTT  | TTCCCACCGC | GGTCCGCAGC | CCACCCGTCC  | 2520 |
|    | TGCTCGGGCC | GCCTTCCTGG  | TCCGGACCGC | GAGTGCCGAG | AGGGCAGGGC | CGGCTCCGAT  | 2580 |
|    | TCCTCCAGCC | GCATCCCCGC  | GACGTCCCGC | CAGGCTCTAG | GCACCCCGTG | GGCACTCAGT  | 2640 |
| 15 | AAACATTTGT | CGAGCGCTCT  | AGAGGGAATG | AATGAACCCA | CTGGGCACAG | CTGGGGGGAG  | 2700 |
|    | GGCGGGGCCG | AGGGCAGGTG  | GGAGGCCGCC | GGCGCGGGAG | GGGCCCCTCG | AAGCCCGTCC  | 2760 |
| 20 | TCCTCCTCCT | CCTCCTCCGC  | CCAGGCCCCA | GCGCGTACCA | CTCTGGCGCT | CCCAGGCGGG  | 2820 |
|    | CCTCTTGTGC | GATCCAGGGC  | GCACAAGGCT | GGGAGAGCGC | CCCGGGGCCC | CTGCTATCCG  | 2880 |
|    | CGCCGGAGGT | TGGAAGAGGG  | TGGGTTGCCG | CCGCCCAGAG | GCGAGAGCGC | CAGAGGAGCG  | 2940 |
| 25 | GGAAGAAGGA | GCGCTCGCCC  | GCCCCCCTGC | CTCCTCGCTG | CCTCCCCGGC | GTTGGCTCTC  | 3000 |
|    | TGGACTCCTA | GGCTTGCTGG  | CTGCTCCTCC | CACCCGCGCC | CGCCTCCTCA | CTCGCCTTTT  | 3060 |
| 30 | CGTTCGCCGG | GGCTGCTTTC  | CAAGCCCTGC | GGTGCGCCCG | GGCGAGTGCG | GGGCGAGGGG  | 3120 |
|    | CCCGGGGCCA | GCACCGAGCA  | GGGGGCGGGG | GTCCGGGCAG | AGCGCGGCCG | GCCGGGGAGG  | 3180 |
|    | GGCCATGTCT | GGCGCGGGCG  | CAGCGGGGCC | CGTCTGCAGC | AAGTGACCGA | GCGGCGCGAC  | 3240 |
| 35 | GGCCGCCTGC | CCCCTCTGCC  | ACCTGGGGCG | GTGCGGGCCC | GGAGCCCGGA | GCCCCGGTAG  | 3300 |
|    | CGCGTAGAGC | CGGCGCGATG  | CACGTGCGCT | CACTGCGAGC | TGCGGCGCCG | CACAGCTTCG  | 3360 |
| 40 | TGGCGCTCTG | GGCACCCCTG  | TTCCTGCTGC | GCTCCGCCCT | GGCCGACTTC | AGCCTGGACA  | 3420 |
|    | ACGAGGTGCA | CTCGAGCTTC  | ATCCACCGGC | GCCTCCGCAG | CCAGGAGCGG | CGGGAGATGC  | 3480 |
|    | AGCGCGAGAT | CCTCTCCATT  | TTGGGCTTGC | CCCACCGCCC | GCGCCCGCAC | CTCCAGGGCA  | 3540 |
| 45 | AGCACAACTC | GGCACCCATG  | TTCATGCTGG | ACCTGTACAA | CGCCATGGCG | GTGGAGGAGG  | 3600 |
|    | GCGGCGGGCC | CGGCGGCCAG  | GGCTTCTCCT | ACCCCTACAA | GGCCGTCTTC | AGTACCCAGG  | 3660 |
| 50 | GCCCCCTCT  | GGCCAGCCTG  | CAAGATAGCC | ATTTCTCAC  | CGACGCCGAC | ATGGTCATGA  | 3720 |
|    | GCTTCGTCAA | CCTCGGTGAG  | TAAGGGCAGG | CGAGGGTACG | CCGTCTCCTT | TCGGGGGCAC  | 3780 |
|    | TTTGAGACTG | GGAGGGAGGG  | AGCCGCTTCT | TCTATGCAGC | CCGCCAGCT  | TTCCGCTCCT  | 3840 |
| 55 | GGCTGAAATC | GCAGTGCCCTG | CCCGAGGGTC | TCCCACCCAC | AGCCCTATGA | CTCCAAGCT   | 3900 |
|    | GTGTGCGCCC | CCAGGTCGGG  | CCGCTGGGTC | GGTGAGCCTG | TAGGGGTTAC | TGGGAAGGAG  | 3960 |
| 60 | GGATCCTCCG | AAGTCCCCTC  | CATGTTACGC | CGCCGGCCGC | ATCTCTGGGG | CTGGAGGCAA  | 4020 |
|    | GGGCGTTCAA | AGCGCGGGGC  | TCGGTCATGT | GAGCTGTCCC | GGGCCGGCGC | CGGTCCGTGA  | 4080 |
|    | CCTGGATGTA | AAGGGCCCTT  | CCCGGCGAGG | CTGCCTTGCC | GCCCTTCCTG | GGCCCCCTCTC | 4140 |
| 65 | AGCCCTGCCT | GGCTCTGGCA  | TCGCGGCCGT | CGCACCCCTT | TACCCTCCCT | GTCAAGCCCT  | 4200 |

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|    |            |            |             |            |            |            |      |
|----|------------|------------|-------------|------------|------------|------------|------|
|    | ACCTGTCCCC | TCGTGGTGCG | CCCGCCTTAG  | GCTACCGGCC | GCTCCGAGCC | TTGGGGCCCC | 4260 |
|    | TCTCCGGGCG | CCGATGCCCC | ATTCTCTCTT  | GGCTGGAGCT | GGGGAAGAAA | CGGTGCCATT | 4320 |
| 5  | GCTAATTTTC | TTTGTTTTCT | TTCTTTGTTT  | ATTTTTTTCT | TTTTTCTTTT | TTTTTCTTTT | 4380 |
|    | CTTTTCTTTT | CTTTTTTTTT | TTTTTTGAGA  | CGGAGTTTCA | CTCTTGCTCG | CCCAGACTGG | 4440 |
| 10 | AGTGCAATGG | CGCGATCTCT | GCTCACCAGCA | ACCTCTGCCT | CCCGGGTTCA | AGCGATTCTC | 4500 |
|    | GTGCCTCAGC | CTCCCGAGTA | GCTGGGATTA  | CAGGCATGCG | CACCATGCCT | GGCTAATTTT | 4560 |
|    | GTATTTTAGT | AGAGACAGGG | TTTCTCCATG  | TTAGGCAGGC | TGGTCTCGAA | CTCCCGATCT | 4620 |
| 15 | CAGGTGATCC | TCCCGCCTCA | GCCTCCCAA   | GTGGTGCTGG | GATTACAGGC | GTGAAGCTGT | 4680 |
|    | GCCCTGCCGC | TAGTCTTCTA | TTTTAAGTAT  | TTAGTGGTAG | GTCCCGGGCC | GGCAGAATCT | 4740 |
| 20 | ATTTTCAGCA | TTTACCACGT | GTGGCGCGCA  | AACCACAGGT | TTTGGCGATT | GGGTGCGCG  | 4800 |
|    | GGATCTCAGA | GCTGACGACC | GCGGGGGCCT  | GGGGGTCCCG | GTTTCCGACT | GGAGCCGCGA | 4860 |
|    | CGACCCCGGC | GACGGCAGCC | TGGGGCTGCA  | GCCGAGGGCC | GGGGAGCTCC | CCCTCCATAT | 4920 |
| 25 | GTGCGCGCAC | ATTCTCCAGA | CTTGCTCAAA  | CTAACCCCC  | GGAGCAGCGC | ACGGGCTGGG | 4980 |
|    | ACTGATGATC | AAATATTTGG | TTTCCGAGAT  | AACACACCCC | GATAGCGCTG | TTTCTGAGC  | 5040 |
| 30 | CGCTTTCATT | CTACTTGTGT | AACCTGCTGC  | GAAAACCCGA | ACCAAGTCAA | GACAGCAAAC | 5100 |
|    | TCACGCCAC  | GGGCCTGTGT | CAACATGGAA  | ATAATGATAC | TGAAGCCCCA | CGCTGGGCAC | 5160 |
|    | CTGGGGCGTG | GACTGGGGGC | GCGGGGGAAG  | CGCAGATCCG | CCTTCATGCT | TCCCTCCTC  | 5220 |
| 35 | CTGATAAGGT | CCCTGGAGTT | CCCGGGAGCC  | ATTGTCTGTA | CTTAATAATA | ACTAAATCCA | 5280 |
|    | ACTAGTGAAC | CAAGCTTCAG | CGAGGCAAGG  | GGAGGGAGGT | TTAGATGCCA | AAATTACCTT | 5340 |
| 40 | CAAAAAAGTT | TAAATTATAC | TAAGCAGCCA  | GTTAAGAAGG | AAGCAGCAAT | ATATGACCTG | 5400 |
|    | ATTTAGAACC | ATCTCCAAGA | TGTATGAGGT  | GGAAAGAAGC | AAGGTGCAGA | TGAGTGGGCT | 5460 |
|    | GCATGTGTGC | TTGTATATCA | TCGTGTCCTC  | CTGGAGGAAG | ACACCAGGAA | CTGGAGAGAG | 5520 |
| 45 | ATTTTACTGG | AGGGGTATAT | GGCGGGGGCA  | TAGCTGGGGC | TTACGGAGTG | GGAGGTGGGG | 5580 |
|    | TCTGATTTTT | CGTCGTCTGC | ACTTCTGTAT  | TTGTGATTTT | TTTAAAACAA | TGTGTATTTA | 5640 |
| 50 | TTAACTATAC | CAAAAAATAA | AGGAAAATTC  | CAAATACATA | CATATAAATA | ATGAACCGCA | 5700 |
|    | GAGCTCTGTC | GCCCTCCTGA | AGCCTGGGGT  | TAGCCAGGGC | CCTTTCTCTG | GTGGGGGATT | 5760 |
|    | TATAGCATCT | TCCCTTCTGT | TGGGTACCCC  | GGACTCCCAC | TGAATGTGCA | GGTCCCAGTG | 5820 |
| 55 | GCTGCCTTCA | GAGCCTGGCT | GGAATCATT   | AAAAGGTATT | TGTAATCTCT | GGCTTCTGCA | 5880 |
|    | GAAGGCCCTG | CAAACCAAGA | GCAAAAAAGC  | CCCCAGTGCT | TATGGGCCGG | CAGTGTGGGC | 5940 |
| 60 | TAGGCCCGGG | GCTCCCTGTC | CCCAAGAGAA  | AGACCAGGTT | GCTCGGAGGG | TGCCTCTGGG | 6000 |
|    | AACTTTGGTG | CGGGCTATTT | GCTCCCCCCA  | TGGCGGCAGG | AGCAAGCTGG | GACTTGTTTG | 6060 |
|    | GGAAGGCCAC | AGCTGGGTGG | TTTTCCTCCT  | CTGGCTGTAC | ATACACCTTT | CAATCCATTT | 6120 |
| 65 | CTTTCATCTT | GAAAGGACAA | AGACCGGCTT  | GTCTGAGCCT | CTTAATCAGT | CAGGCTGGCT | 6180 |

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|    |             |            |            |            |            |            |      |
|----|-------------|------------|------------|------------|------------|------------|------|
|    | TTGGGCTTTG  | GGGACCCTGA | CTTTCTCAGG | TCTAGCTTTC | TGGGACATCA | CTCCAAATTA | 6240 |
|    | GATGGCAGAG  | TGGCTTTTAA | CAGAGCGCAC | TGACCTTGTT | TTCTTTCTCT | CTCTGTCCCT | 6300 |
| 5  | AAACTCGAGG  | TCATTAGTTA | GGTGAAGACC | TGGGCTGCAG | TTTGGCGAGA | CACTTCCTGT | 6360 |
|    | AGATGCTTCT  | AATGTTGGCC | TTTAATTTCT | GCTAAGCAGC | AGCACACAAA | TAAATGGCCT | 6420 |
| 10 | GTCCCTTCTA  | TCCTGTTGTA | GCTTGGAATT | TCTCCATAGG | AGGGACTTGG | GGGTGGCAGT | 6480 |
|    | AGGGTTGGAG  | AGGGTTGGGG | GGAGGTGTAG | GAGACTTGTC | TGGCCACTGA | GTTTGCTGAG | 6540 |
|    | AAAGTACTGC  | TATAGTGTTC | TTCCTTGGAT | TGCAAATCAT | GTTGATCTGA | ACTGCTGATT | 6600 |
| 15 | TGAAGTGGAT  | TGAGAGGATG | GAACAATAGA | AGGAGGATAT | GGCTCAGGAC | AGTCAAGTAC | 6660 |
|    | TGGAAGAGGG  | AAAGGTACAA | AGAGGTGTTG | GCACTGAATG | ACCCTGAACA | GGGCTGCCCT | 6720 |
| 20 | GGAAATATCA  | GAGGTGAGTG | ACAAAGAGAA | CTCTAGTCGA | AGGTCTGGAA | GTCAATTATT | 6780 |
|    | GTCTCCAGCT  | TTTGTCCAC  | CCTAAGGGAT | GGAGCATGAA | CTTCATGCAT | GTAACATCCC | 6840 |
|    | TCCAGGAGCG  | CTGAGGTTCT | GGGAATTCCC | AGTGCTGGCT | ACCATGCCAT | TCTTTTCTCA | 6900 |
| 25 | TTCACTCAAG  | AGCGTATTGG | GATATGCGTG | CATGAAAGCA | ATGTAATTAT | GGGCACAACC | 6960 |
|    | TCAAAACCTG  | CTCTAATTTT | TTTTTTTTTT | GGAGATGGAG | TCTCGCTCCA | TCACCCAGGC | 7020 |
| 30 | TGGAGTGCAA  | TGGCGCGATC | TCAGCTCACT | GCAAGCTCAG | ACCTCCAGGG | TTCACACCAT | 7080 |
|    | TCTCCTGCCT  | CAGCCTCCCG | AGTAGCTGGG | AATACAGGCG | CCCGCACCAT | GCGCGGCTAA | 7140 |
|    | TTTTTTTGTA  | TTTTTAGTAG | AGACGGGGTT | TCACTGTGTT | AGCCAGGATG | GTCTCGATCT | 7200 |
| 35 | CCTGACCTCG  | TGATCCACCC | GCCTCGGCCT | CCCAAAGTTC | TGGGATTACA | GGCGTGACAG | 7260 |
|    | CCGTGCCCCG  | AATCTGCTCT | AATTTTTTAA | AGATATCATT | TGCAAACCTT | GGGCACTTGA | 7320 |
| 40 | GTCACCTCAGT | AAGATATTAT | TTACAACCCC | ACCATAGATT | CAAACCTCTG | TCCTAGAATG | 7380 |
|    | TTGTCGAGTT  | AGGCATCTGG | CTTGACGAA  | CAGCTGGCTT | TCCTGTCTAT | GCTGTCTCCT | 7440 |
|    | TCCAGGGAGG  | ATGTTTCACC | CTTCATATTG | AGGAAATGGG | CACAGAGAAC | CCATTTCTCT | 7500 |
| 45 | TACTCATCAT  | GTAACCTCAG | TGGGATGGTC | AGATCTATCT | TTAACCTGGC | CACTCTTCCA | 7560 |
|    | CAAGCTCACA  | CTGACTCCAG | CAAGATCTTA | AACTAGAAGG | CAGGAGTTCA | AATCCTAGCT | 7620 |
| 50 | GGTGCACTGG  | CCAAATCTCG | GCTCACAGCA | CCTTCTGCCT | CCTGGGCTCA | AGCGATCCTC | 7680 |
|    | TGACCTCAGT  | CTCCCAAGTA | GCTGGGACCA | TAGGCATGCA | CCACTATGCC | TGGCTAATTT | 7740 |
|    | TTGTATTTTT  | GTAATTTTTT | GTAGAGACAG | AGTTTCACCA | TGTTGCCCAG | CCCAGTCTTG | 7800 |
| 55 | AACTCCTGGA  | CTCAAGCAAT | CTTCCCACCT | TTGCCTACCA | GAGTGCCGGG | ATTACAGGTG | 7860 |
|    | TGAGCCATCA  | TGCTAGTTGC | GCACAGTTGG | GCGAAACTGA | CAGATGAGAA | AGCAGAACCT | 7920 |
| 60 | CGTGAGTCCA  | CTCAGTAAGA | GACTCCCTAC | TTTCTTTCTG | AGTCTTTGTT | TCTCATCAAT | 7980 |
|    | TGAATGGCAA  | TAAACAACCT | GGTGGCCCAA | GAGTTGATGA | CAACAGTCCT | ATAAGATTAT | 8040 |
|    | ACATGTAAAA  | GAAACAGAGT | ATTCTACAAA | TATCAGTTAT | TGATAGTTCA | ATAGGCAACC | 8100 |
| 65 | TGACATTACC  | TTTTCTTGGA | ACTTGATGAA | CAACTCAGAA | ACTCATTAAT | ATCAAACCCA | 8160 |

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|    |                                                                    |       |
|----|--------------------------------------------------------------------|-------|
|    | ATGGTGAGCA CTTGGTCTTT ATTTATGGCT GTAAGAGAAG AAATTGAATT AACTCTATGT  | 8220  |
|    | AAATGCCAAC TAAGAACATC GAAGTCTGAA ATCAACAGTT TTCCTCGCTC ATACGACACA  | 8280  |
| 5  | CCCAAACCTCA AGCAGTGGTT CCAAGCCCCT TTGGAAAATA CCATGGGCTA ACGACTTTAA | 8340  |
|    | AAGCTTAGAA GTGAATTCTA CTTACTTATT ACTTAAAAGT GGTTCCTCAA CTTCAAGGTG  | 8400  |
| 10 | AATCAAAATC ATCTGTAGAG CTTGTAAAAA CACAGGTTGC TGGTCCACCC CAAGAGTGTC  | 8460  |
|    | TTGAGTCAGT AGGTCTCAAG TAGGGCTCAA GAATATGCAT TTCTAATGAG CTCCAGGTGA  | 8520  |
|    | GTCTAAGTGT TAGTCGTCGG TCTTGGGACC ACAACTTTGG GAACAATTGA TTTAGAAGAA  | 8580  |
| 15 | CTCAAAGATC AGAAAGGGGT GGAATATTTT TAAAATTGTG GTAAAATACG CATAAACAGA  | 8640  |
|    | AAAGGTACAA TTTTAACCAC TTAGAGAGAG GTGGGATCTA AGAACAGAAA TTGTTATGCC  | 8700  |
| 20 | ATCAAAGGTG AGTTCAGATA AGCATTATTA AATGGTATCT ATGGATAAAC TTCAGGGGCC  | 8760  |
|    | CTGTGGAGCA ACCCAATGCT GGGATGGGGT CCAGGTGTGC TATGGTTTGG ATGTGGTTTG  | 8820  |
|    | TCCCTACAAA AACTCATGTT GAAATTTAAT TGCCAGTGTA ACATTATTGA GAGGTTATGG  | 8880  |
| 25 | ACTTTTAAGA GGCATTTGGG TCATGAGGGA TCCACCTTCA GGGATTAGTG CAGTCTCCAG  | 8940  |
|    | GGAGTGAGTG AGTTCCCATT CTAGTGGGAC TGGATTAGTT ACCATACAGT GGTGTGTATA  | 9000  |
| 30 | AAGTGAGGCT GCTTCTGGTG TTTTATCTGT TTGCAGGCAC TTCCTTCCCC TTCCACTTCT  | 9060  |
|    | CTGCCAGGTT AGGATGCAGC ATGAGGCCCT CACCAGAAGC TGACCAGATG TGGCTGCCTG  | 9120  |
|    | ATCTTGAACT TCCCAGTCCC CAGAACCATG AGCTAAATAA ACCTTTTTTC TCTATAAATT  | 9180  |
| 35 | ACGCAGTCTA GAGTATTCTA TTATAGCAAC ACAAGACAGA CTAAGACACA GTGGTAGAAA  | 9240  |
|    | GAACACTACT GACTTCTCCC ATACTCTGGC CTATGGACAA GAGTGACAGA CAGACAAGAG  | 9300  |
| 40 | TGAATATCAG GGCCCTCAGG CACATTCCCTC TCTGCCCTT CCTCCCTTCT TGCAGAGTCT  | 9360  |
|    | CCAGTGACTG CCAGCTAATG CTATCATAGA CCCACCTTT CCCCTGACTT GATTGGACCA   | 9420  |
|    | GAAGCAGCCT CCTGATCCAT GGCCAACAAT CAGATTCACT TTCAAGAATT TGAACATAAGA | 9480  |
| 45 | GACACTAGGA AGATGGCCCT TGAGCTGTGA GTCCTACACT TGAAAGTTCT TAGCATCTTG  | 9540  |
|    | GTCAGGTACC CACCAGGGCC ATGTGCAAAC TGAGATAATG GGGACATGGA ACAAGGGTAA  | 9600  |
| 50 | GTGGAGAGGG CTGGCTGGAG AGAGACGGGC AGAGGAAAGC CCTGCCAAGA GGAGCAGAGA  | 9660  |
|    | TGAGAGACCT TGGAGGGAGA GGTAATAAAA GGAGGCAAAG ATGATTTTCC ATGCTTACAA  | 9720  |
|    | CTCACAGCTG AGGCCTAACT ATCTTTATGT CCATAAGAGG CATCCTTG TGGAACCTCT    | 9780  |
| 55 | CCTCTTTCTT GGGTCAATGG GGGATGGTTG CAAGGGACCA TCAGTAGGAA GGCATAGTAC  | 9840  |
|    | ACTAACCAG TCTGGGGTGG GCTTTTAGAC TAGTCTTCCT CCCATGCTCC TCCTCCCAT    | 9900  |
| 60 | GGAACCCCGG ACTTTCAAGA CTGCTACCTA GCACACCAGT GCACCAGATG TCACTCAAAA  | 9960  |
|    | CCTCTTCAGC AATGGCCAC TCACCTTCAA AAAGGCTGAA GAGCAGACTG GCTGGGTTCT   | 10020 |
|    | TCATGGTGGA GGGGCAGTCT GGGAGGTTTT AAGGTTGAAG ATGAAAACCT TCACTTTTGG  | 10080 |
| 65 | CTCAATGGTC TGAAAAAGAG AAGGACCAGC AAGTGAAGT AAGCCTCCTG GAAAGCATCT   | 10140 |

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|    |            |            |            |             |            |            |       |
|----|------------|------------|------------|-------------|------------|------------|-------|
|    | TGATAACAGG | GGCAGAGTTT | CAAGATGAGA | AGCTGTGGCA  | CTTACTCTGG | CTTTGGAAAT | 10200 |
|    | GACCTCTAAG | TATCTCAGTT | AATTAAAGGA | GTCAAACCTCT | AGACTCGAAG | GAGAAGATCT | 10260 |
| 5  | ACAATTTTCA | ATAACATAGT | CTACCCTCCC | CTCCTTCCCC  | CACCTTCACC | TCTTCTTTCA | 10320 |
|    | TCACAGGCTT | ACAGGGCACC | TCTTAGAGCC | AGGCACGGTG  | TTGGGATCAG | GAACAAGGCC | 10380 |
| 10 | ACTGCTCACA | TCCAGAGCCT | GTGCTACTTA | AGAAGCTTCC  | AGGACCTCTT | GGATGGCTGT | 10440 |
|    | GGTTAGTGCC | CTACTTTTCC | CAGCAGGTTG | GATGCAGAAT  | CATGCTCTTG | TCGTTCAGGA | 10500 |
|    | TGACCATGGG | GACCATGGGT | CTGAGCCTGT | GACCCTCCAG  | TCTACAGTGT | GTTGGTGAGG | 10560 |
| 15 | AAGGAGCAGT | TGTCACTGGG | GTCACTGGCA | ATGGGCATGC  | CTCCATCTAG | CTTAGGCAAG | 10620 |
|    | ATGCTTAGAC | TCAGAGCCAG | AGAGTGAAAC | CCAGACACTA  | ATGAGCTGTC | GGTGTGGTG  | 10680 |
| 20 | TGTGTTCTCT | TCCTCTTCCA | GTGGAACATG | ACAAGGAATT  | CTTCCACCCA | CGCTACCACC | 10740 |
|    | ATCGAGAGTT | CCGGTTTGAT | CTTTCCAAGA | TCCCAGAAGG  | GGAAGCTGTC | ACGGCAGCCG | 10800 |
|    | AATTCGGGAT | CTACAAGGAC | TACATCCGGG | AACGCTTCGA  | CAATGAGACG | TTCCGGATCA | 10860 |
| 25 | GCGTTTATCA | GGTGCTCCAG | GAGCACTTGG | GCAGGTGGGT  | GCTATACGGG | TATCTGGGAG | 10920 |
|    | AGGTGCTGAG | TTTCTCTGG  | GGGCAGAGGA | AGAAGGTGGT  | GAGGGTTTCC | CTCCCCTCCC | 10980 |
| 30 | ACCCCATGAG | CTCTGCTTCC | CATCTGTTGG | GGTAGTGGAG  | CTGTGACCTG | CTAACGCGAA | 11040 |
|    | GCCCGTGTCT | CTCCTCCTCT | CTCGCAGGGA | ATCGGATCTC  | TTCTTGCTCG | ACAGCCGTAC | 11100 |
|    | CTCTGGGCCT | CGGAGGAGGG | CTGGCTGGTG | TTTGACATCA  | CAGCCACCAG | CAACCACTGG | 11160 |
| 35 | GTGGTCAATC | CGCGGCACAA | CCTGGGCCTG | CAGCTCTCGG  | TGGAGACGCT | GGATGGTGAG | 11220 |
|    | TCCCCCGCCA | CTGCCAGTCC | TAATGCAGCC | TGTGCTCCTG  | GACTTCAGGA | GGGTCTCAGC | 11280 |
| 40 | AGTGCTCATG | CTTGCTTCAC | TACAAACAGG | CTTCCCCGCC  | CCTCCCAACC | AGTACTCCAT | 11340 |
|    | GTTCAGCCTT | TTGATCCTGC | AGCCCTGTCC | CGCTCGTGCC  | CCTCCTGTAA | CTGCTCTTCT | 11400 |
|    | GTGCACTTGG | CTGCTTCCTG | TCCAGGGCAG | ACGATCAACC  | CCAAGTTGGC | GGGCCTGATT | 11460 |
| 45 | GGGCGGCACG | GGCCCCAGAA | CAAGCAGCCC | TTCATGGTGG  | CTTCTTTCAA | GGCCACGGAG | 11520 |
|    | GTCCACTTCC | GCAGCATCCG | GTCCACGGGG | AGCAAACAGC  | GCAGCCAGAA | CCGCTCCAAG | 11580 |
| 50 | ACGCCCAAGA | ACCAGGAAGC | CCTCGGATGG | CCAACGTGGC  | AGGGTATCTT | AGGTGGGAGG | 11640 |
|    | GATCACAGAC | CCACCACAGG | AACCCAGCAG | GCCCCGGCGA  | CCGCAGGAGA | CTGACTAAAA | 11700 |
|    | TCATTCAAGT | CTACCAAGA  | TGCTCTGAGC | TCTCTTCGAT  | TTTAGCAAAC | CAGGAGTCCG | 11760 |
| 55 | AAGATCTAAG | GAGAGCTGGG | GGTTTGACTC | CGAGAGCTCG  | AGCAGTCCCC | AAGACCTGGT | 11820 |
|    | CTTGACTCAC | GAGTTAGACT | CCACTCAGAG | GCTGACTGTC  | TCCAGGGTCT | ACACCTCTAA | 11880 |
| 60 | GGGCGACACT | GGGCTCAAGC | AGACTGCCGT | TTTCTATATG  | GGATGAGCCT | TCACAGGGCA | 11940 |
|    | GCCAGTTGGG | ATGGGTTGAG | GTTTGGCTGT | AGACATCAGA  | AACCAAGTC  | AAATGCGCTT | 12000 |
|    | CAACCAGTAG | AAAATTCACC | AGCCCGCAGA | GCTAAGGTTG  | GGTGGACATT | AGGGTTGGTT | 12060 |
| 65 | GATCCAGGAG | CTCAACAGTG | TCCTCTGAGC | CCCAGCTCCT  | TCTGCCCCAC | CCCACCATCT | 12120 |

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|    |             |            |             |            |             |            |       |
|----|-------------|------------|-------------|------------|-------------|------------|-------|
|    | TCAGTGCTGC  | TTCCTCTCAA | GGCCACAGCT  | GTAGTTGGCC | AGGGGGGCTT  | CATTATTTTT | 12180 |
|    | TGCTCCTGGG  | CAGTAGGAGG | AAGAGAATGA  | ATGTCTCTCC | ATGGGTCTTT  | CTTAGGAATG | 12240 |
| 5  | TGGGAAC TTT | TTCCAGAAGT | CTCTATGTCT  | TTTAGTTTGT | GTTGGGTCAC  | TTGCCCTTCC | 12300 |
|    | TGAACCACTT  | CCTGACTCCT | GGACAGGATG  | TGCACTGATG | AGCTTAGCTT  | TGGGGATCTA | 12360 |
| 10 | ATAGTGACTT  | TACAAAGCCT | CTTTGAGAAG  | GTGACATTGG | AACCAAGGCT  | TGAGCAGACA | 12420 |
|    | CAACAAAGAT  | TGCAGGGAGG | GGCATTGCAG  | GTGGAGGAAA | CGGCACATGC  | AAGAGCCCTG | 12480 |
|    | CGTGGGAGTG  | AGCTTGGTGT | TTGGTCAATC  | AGTTGTCAGA | GCACACCGGG  | CCCTGTCAGC | 12540 |
| 15 | AGGCACAGCC  | TGGGCCTGCT | CTGAGTATGA  | CAGAGAGCCC | CTGGGAAGTT  | GTAGGTGGAG | 12600 |
|    | GAAAGACAGG  | TCATGACTAG | GAAAAAAGCA  | ATCCCTCTGT | TGTGGGGTGG  | AAGGAAGGTT | 12660 |
| 20 | GCAGTGTGTG  | TGAGAGAGAG | ACAAGACAGA  | CAGACAGACA | CTTCTCAATG  | TTTACAAGTG | 12720 |
|    | CTCAGGCCCT  | GACCCGAATG | CTTCCAAATT  | TACGTAGTTC | TGGAAAACCC  | CCTGTATCAT | 12780 |
|    | TTTCACTACT  | CAAAGAAACC | TCGGGAGTGT  | TTTCTTCTGA | AAGGTCATCA  | GGTTTTGACT | 12840 |
| 25 | CTCTGCTGTC  | TCATTTCTTC | TTGCTGGTGG  | TGGTGATGGT | TGCTTGTCCC  | AGGCCCTGTC | 12900 |
|    | CCGCATCCTC  | TTGCCCCGTC | AGAGGGATGA  | GTGTGTTGGG | GCCTCACGAG  | TTGAGGTTGT | 12960 |
| 30 | TCATAAGCAG  | ATCTCTTTGA | GCAGGGCGCC  | TGCAGTGGCC | TTGTGTGAGG  | CTGGAGGGGT | 13020 |
|    | TTCGATTCCC  | TTATGGAATC | CAGGCAGATG  | TAGCATTTAA | ACAACACACG  | TGTATAAAAG | 13080 |
|    | AAACCAGTGT  | CCGCAGAAGG | TTCCAGAAAG  | TATTATGGGA | TAAGACTACA  | TGAGAGAGGA | 13140 |
| 35 | ATGGGGCATT  | GGCACCTCCC | TTAGTAGGGC  | CTTTGCTGGG | GGTAGAAATG  | AGTTTTAAGG | 13200 |
|    | CAGGTTAGAC  | CCTCGAACTG | GCTTTTGAAT  | CGGGAAATTT | ACCCCCCAGC  | CGTTCTGTGC | 13260 |
| 40 | TTCATTGCTG  | TTCACATCAC | TGCCTAAGAT  | GGAGGAACTT | TGATGTGTGT  | GTGTTTCTTT | 13320 |
|    | CTCCTCACTG  | GGCTCTGCTT | CTTCACTTCC  | TTGTCAATGC | AGAGAACAGC  | AGCAGGCACC | 13380 |
|    | AGAGGCAGGC  | CTTGTAAGAA | GCACGAGCTG  | TATGTCAGCT | TCCGAGACCT  | GGGCTGGCAG | 13440 |
| 45 | GTAAGGGGCT  | GGCTGGGTCT | GTCTTGGGTG  | TGGGCCCTCT | GGCGTGGGCT  | CCCACAGGCA | 13500 |
|    | GCGGGTGCTG  | TGCTCAGTCT | TGTTTCTCAT  | CTCTGCCAGT | TAAGACTCCA  | GTATCAAGTG | 13560 |
| 50 | GCCTCGCTAG  | GGAAGGGGAC | TTGGGCTAAG  | GATACAGGGA | GGCCTCATGA  | AATCCGAGAG | 13620 |
|    | CAGAAATGTG  | GTTGAGACTT | GAAC TCGAAC | CAGGAACCCA | AACACTTTGG  | ACTCTGAACC | 13680 |
|    | CCATTCTCTG  | CATGCACCTC | ATTCCCATCC  | CTTGGCTGGC | TGCTTCTCAA  | GATGATGCCG | 13740 |
| 55 | GGCCGTGTGT  | TTGAATGTAG | ATACCTGGGG  | AGCCATCTCC | CCCTCTGCCC  | TCTGACTTCA | 13800 |
|    | TTTACCCCAT  | TCCCATTCCC | ACGGGAGGGA  | CGGATCTCCC | CAGCTTGGTT  | CAGGCGCTTG | 13860 |
| 60 | TTCCTGAACC  | AGTCAACTGT | TTCAGGGGTG  | GGGTCATGTT | ACTGGCACAT  | GGCTGCCCCC | 13920 |
|    | TCTGGAGCCA  | TTTGCATGGA | GTGAGGCAAA  | AGGCAGGGGA | TGAATCTAGG  | AGAGGAGTGA | 13980 |
|    | GGGTCATGTG  | ATCCACCTGC | CGTGAGCTCT  | GGATCGTGAT | TCTCATT CAG | CAGTCACGAG | 14040 |
| 65 | CATCTCGAGC  | GTTCTGGGCC | CTGTTCTAGG  | TACTGGATTG | GAGATGCAGC  | GATGAACACT | 14100 |

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|    |            |            |            |             |            |            |       |
|----|------------|------------|------------|-------------|------------|------------|-------|
|    | GCAATGTGTC | TGCCCTGTGG | GGCTCAAATA | TCCCTGGAGA  | GGGTATTGTC | ATGAGGTCAT | 14160 |
|    | CAGGGCAACT | GGTGGTATTC | TACCCTCAGG | GAGCTTGTAG  | TTCAGTGGGA | GAGTCCAGAA | 14220 |
| 5  | TCTTCCCTGG | GGATTATGCC | CAGACACACT | CAGGGCGTAC  | GTGCACACAG | CCAGCTCTGA | 14280 |
|    | GCCCTCCTGT | GAGCCTGCCC | TCAGGACTGA | TGACCACATC  | TACCTGCAGC | TGGGACAGAA | 14340 |
| 10 | CCCAAACCTC | AGGGGCCTCT | GCTGGAAGAT | TCCATGTGCT  | TAAGCATCAC | TGAGGAGTAT | 14400 |
|    | ATTGATTATT | GGGCAACATT | TCTGTGCCAC | CCAGACCCTA  | GAGGCAAGGA | TGGCACATGG | 14460 |
|    | ATCCCTTACT | GACCAGTGCA | CCCGGAGCCA | GCATGGGTGA  | TGCCATTATG | AGTTATTAGC | 14520 |
| 15 | CTCTCTGGCA | GGTGGGCAAA | CCGAGGCATG | GAGGTTTGT   | TAAGGTGAAC | TGCCAGTGTG | 14580 |
|    | TGACCACCTA | GTGGGGGTAG | AGCTGATGAT | TGCCTCACAC  | CGGAGGCTCC | TTCCTGTGCC | 14640 |
| 20 | GCGTTCTGTC | CAGAAGACAC | AGCCATGGAT | GTCCATTTTA  | GGATCAGCCA | AGCCCGTGGG | 14700 |
|    | GCTTTCCTTC | ATTTTTATTT | TATGTTTTTT | TAGAAATGGG  | GTCTTGCTCT | GTCACCCAGG | 14760 |
|    | CTGGGGTGCA | GTGGTGTGAT | CATACGTCAC | CGCAGCTTTG  | AGCCGTCTTC | CCACTCAGTC | 14820 |
| 25 | TACTAAGCTT | GGACTATAGG | CCAAGACTAT | AGAGTGGTCC  | TTCTTTCCAT | TCTTTTGGGA | 14880 |
|    | CCATGAGAGG | CCACCCATGT | TTCCTGCCCC | TGCTGGGCCC  | TGCTGCTCAG | AAGGCATGGT | 14940 |
| 30 | CTGAGGCTTT | CACCTTGGTC | GTGAGCCTTC | GTGGTGGTTT  | CTTTCAGCAT | GGGGTTGGGA | 15000 |
|    | TGCTGTGCTC | AGGCTTCTGC | ATGGTTTCCC | ACACTCTCTT  | CTCCTCCTCA | GGACTGGATC | 15060 |
|    | ATCGCGCCTG | AAGGCTACGC | GCGCTACTAC | TGTGAGGGGG  | AGTGTGCCTT | CCCTCTGAAC | 15120 |
| 35 | TCCTACATGA | ACGCCACCAA | CCACGCCATC | GTGCAGACGC  | TGGTGGGTGT | CACGCCATCT | 15180 |
|    | TGGGGTGTGG | TCACCTGGGC | CGGGCAGGCT | GCGGGGCCAC  | CAGATCCTGC | TGCCTCCAAG | 15240 |
| 40 | CTGGGGCCTG | AGTAGATGTC | AGCCCATTCG | CATGTCATGA  | CTTTTGGGGG | CCCCTTGCGC | 15300 |
|    | CGTTAAAAAA | AAATCAAAAA | TTGTACTTTA | TGACTGGTTT  | GGTATAAAGA | GGAGTATAAT | 15360 |
|    | CTTCGACCCT | GGAGTTCATT | TATTTCTCCT | AATTTTAA    | GTAATAAAAA | GTTGTATGGG | 15420 |
| 45 | CTCCTTTGAG | GATGCTTGTA | GTATTGTGGG | TGCTGGTTAC  | GGTGCCTAAG | AGCACTGGGC | 15480 |
|    | CCCTGCTTCA | TTTTCCAGTA | GAGGAAACAG | GTAAACAGAT  | GAGAAATTTC | AGTGAGGGGC | 15540 |
| 50 | ACAGTGATCA | GAAGCGGGCC | AGCAGGATAA | TGGGATGGAG  | AGATGAGTGG | GGACCCATGG | 15600 |
|    | GCCATTTCAA | GTAAATTTTC | AGTCGGGTCA | CCAGGAAGAT  | TCCATGTGAT | AATGAGATTA | 15660 |
|    | ACGTGCCCAG | TCACGGCGAC | ACTCAGTAGG | TGTTATTCCCT | GCTCTGCCAA | CAGCAACCAT | 15720 |
| 55 | AGTTGATAAG | AGCTGTTAGG | GATTTTGTCC | TTTGTGCTTAG | AATCCAAGGT | TCAAGGACCT | 15780 |
|    | TGGTTATGTA | GCTCCCTGTC | ATGAACATCA | TCTGAGCCTT  | TCCTGCCTAC | TGATCATCCA | 15840 |
| 60 | CCCTGCCTTG | AATGCTTCTA | GTGACAGAGA | GCTCACTACC  | AGGACTACTC | CCTCCTTTCA | 15900 |
|    | TTTAGTAATC | TGCCTCCTTC | TTTTCTTGTC | CCTGTCCTGT  | GTGTAAAGTC | CTGGAGAAAA | 15960 |
|    | ATCTCATCTA | TCCCTTTCAT | TTGATTCTGC | TCTTTGAGGG  | CAGGGGTTTT | TGTTTCTTTG | 16020 |
| 65 | TTTGTTTTTT | TAAGTGTTGG | TTTTCCAAAG | CCCTTGCTCC  | CCTCCTCAAT | TGAAACTTCA | 16080 |

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AAGCCCTCAT TGGGATTGAA GGTCCCTTAGG CTGGAAACAG AAGAGTCCTC CCCAACCTGT 16140  
TCCCTGGCCT GGATGTGCTG TGCTGTGCCA GTATCCCCTG GAAGGTGCCA GGCATGTCTC 16200  
5 CCCGGCTGCC AGGGGACACA TCTCTATCCT TCTCCAACCC CTGCCTTCAT GGCCCATGGA 16260  
ACAGGAGTGC CATCGCCCTG TGTGCACCTA CTTCCATCAG TATTTACCA GAGATCTGCA 16320  
10 GGATCAAAGT GAATTCTCCA GGGATTGTGA AATGATGCGA TTGTGGTCAT GTTTAAAAGG 16380  
GGGCAACTGT CTTCTAGAGA GTCCTGATGA AATGCTTCCA GAGGAAATGA GCTGATGGCT 16440  
GGAATTTGCT TTAAATCAT TCAAGGTGGA GCAGGTGGGG AAGGGTATGG ATGTGTAAGA 16500  
15 GTTTGAAATT GTCCATCATA AAATGTGTAA AAAGCATGCT GGCCTATGTC AGCAGTCACA 16560  
GCCTGGAGGT GGTAACAGAG TGCCAGTCAC TGATGCTCAA GCCTGGCACC TACAGTTGCT 16620  
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20 AACACGTGGC AGATCTGCTA ACACTGATCT TGGTTGGCTG CCGTCAGCTT AGGTTGAGTG 16740  
GCGGTCTTCC CTTAGTTTGC TTAGTCCCCG CTATTCCCTA TTGTCTTACC TCGGTCTATT 16800  
25 TTGCTTATCA GTGGACCTCA CGAGGCACTC ATAGGCATTT GAGTCTATGT GTCCCTGTCC 16860  
CACATCCTCT GTAAGGTGCA GAGAAGTCCA TGAGCAAGAT GGAGCACTTC TAGTGGGTCC 16920  
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ATGCTTTGTT CCCCTTGTCT GCAAAACAGG GATAATCCCA GAACTGAGTT GTCCATGTAA 17100  
35 AGTGCTTAGA ACAGGGAGTG CTTGGCTTGG GGAGTGTAC CTGCAGTCAT TCATTATGCC 17160  
CAGACAGGAT GTTTCTTTAT AGAAACGTGG AGGCCAGTTA GAACGACTCA CCGCTTCTCA 17220  
CCACTGCCCCA TGTTTTGGTG TGTGTTTCAG GTCCACTTCA TCAACCCGGA AACGGTGCCC 17280  
40 AAGCCCTGCT GTGCGCCAC GCAGCTCAAT GCCATCTCCG TCCTCTACTT CGATGACAGC 17340  
TCCAACGTCA TCCTGAAGAA ATACAGAAAC ATGGTGGTCC GGGCCTGTGG CTGCCACTAG 17400  
45 CTCCTCCGAG AATTC 17415

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

TTCACGCWTS ANTKMNY

17

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs

- 56 -

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

5

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

10

GTCAYGCRTG A

11

(2) INFORMATION FOR SEQ ID NO:4:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 102 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

20

(ix) FEATURE:

25

(A) NAME/KEY: Peptide  
 (B) LOCATION: 1..102  
 (D) OTHER INFORMATION: /note= "Wherein each Xaa is  
 independently selected from a group of one or more specified  
 amino acids as defined in the specification."

30

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

35

Cys Xaa Xaa His Glu Leu Tyr Val Ser Phe Xaa Asp Leu Gly Trp Xaa  
 1 5 10 15

Asp Trp Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly  
 20 25 30

40

Glu Cys Xaa Phe Pro Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala  
 35 40 45

Ile Xaa Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa Xaa Val Pro Lys  
 50 55 60

45

Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa  
 65 70 75 80

Asp Xaa Ser Xaa Asn Val Ile Leu Xaa Lys Xaa Arg Asn Met Val Val  
 85 90 95

50

Xaa Ala Cys Gly Cys His  
 100

(2) INFORMATION FOR SEQ ID NO:5:

55

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 97 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

60

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## (ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..97

(D) OTHER INFORMATION: /note= "Wherein each Xaa is  
independently selected from a group of one or more specified  
amino acids as defined in the specification."

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

```

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1              5              10              15
Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly Xaa Cys Xaa Xaa Xaa
15              20              25              30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
              35              40              45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Pro
20              50              55              60
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
25              65              70              75              80
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys
              85              90              95
Xaa
30

```

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 102 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

## (ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..102

(D) OTHER INFORMATION: /note= "Wherein each Xaa is  
independently selected from a group of one or more specified  
amino acids as defined in the specification."

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

```

Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1              5              10              15
Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly
55              20              25              30
Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
60              35              40              45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
              50              55              60
Xaa Xaa Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65              65              70              75              80

```

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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
85 90 95

Xaa Xaa Cys Xaa Cys Xaa  
100

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..5  
(D) OTHER INFORMATION: /note= "Wherein each Xaa is  
independently selected from a group of one or more specified  
amino acids as defined in the specification."

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

Cys Xaa Xaa Xaa Xaa  
1 5

## THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A vector comprising a DNA sequence encoding a reporter gene in operative association with at least two OP-1-specific non-coding DNA sequences capable of modulating expression of said reporter gene, wherein said non-coding sequences each comprise a different Pax-responsive OP-1 modulating element which is responsive to a different Pax gene expression product.
2. The vector of claim 1 wherein one of said non-coding sequences is selected from the group of DNA sequences corresponding to bases 108-121, 139-154, 157-167, 365-378, 497-511, 598-613, 1123-1140, 1144-1161, 1285-1297, 1750-1762, 2001-2023, 2365-2378, 2931-2944 of SEQ. ID No. 1, including allelic, species and other sequence variants thereof.
3. The vector of claim 1 wherein one of said non-coding sequences corresponds to part or all of SEQ. ID No. 2 including allelic, species, and other sequence variants thereof.
4. The vector of claim 1 wherein one of said non-coding sequences comprises between one and twelve Pax-responsive OP-1 modulating elements.
5. The vector of claim 1 wherein one of said non-coding sequences comprises at least part of a Pax-responsive OP-1 modulating element.
6. The vector of claim 1 wherein one of said non-coding sequences is selected from the group of DNA sequences corresponding to bases 491-503, 737-747, 891-903, 994-1006 of SEQ. ID No. 1, including allelic, species and other sequence variants thereof.
7. The vector of claim 1 where one of said non-coding sequences corresponds to part or all of SEQ. ID No. 3 including allelic, species and other sequence variants thereof.



8. The vector of claim 1 wherein one of said non-coding sequences comprises four or more second Pax-responsive OP-1 modulating elements.
9. The vector of claim 1 wherein one of said non-coding sequences defines at least part of a second Pax-responsive OP-1 modulating element.
10. A cell transfected with a vector as claimed in any one of the preceding claims.
11. The cell of claim 10 further comprising a vector containing at least one non-coding sequence that is a Pax-responsive OP-1 modulating element.
12. A cell comprising a transfected vector, said vector encoding a reporter gene in operative association with at least two DNA sequences,  
the first said sequence comprising the functional protein encoding part or all of a sequence selected from SEQ. ID No. 2, including allelic, species and other sequence variants thereof, and  
the second said sequence comprising part or all of a sequence selected from SEQ. ID No. 3, including allelic, species and other sequence variants thereof.
13. The cell of claim 12 further comprising a transfected vector encoding at least one Pax gene expression product.
14. A method for identifying a candidate compound for the ability to modulate expression of OP-1, said method comprising the steps of:
- (a) incubating a candidate compound with a cell co-transfected with vectors comprising:
    - (i) a DNA sequence encoding a reporter gene in operative association with at least one OP-1-specific non-coding sequence lying contiguous to the OP-1 gene under naturally-occurring conditions which can modulate expression of said reporter gene, wherein said non-coding sequence defines at least one Pax-responsive OP-1



modulating element which is responsive to a first Pax gene expression production; and

- (ii) a DNA sequence encoding a first Pad gene expression product;
- (b) measuring the level of reporter gene expressed in said cell; and
- (c) comparing said level with that of said reporter gene expressed in said cell in the absence of said candidate compound, wherein a change in reporter gene expression level is indicative of said candidate's ability to modulate OP-1 expression.

15. The method of claim 14 wherein step (a) further comprises incubating said candidate compound with a cell co-transfected with DNA sequences encoding a second Pax-responsive OP-1 modulating element, which is responsive to a second Pax gene expression product, in operative association with a reporter gene, and DNA sequence encoding a second Pax gene expression product.

16. The method of claim 15 wherein said first non-coding sequence is selected from the group of DNA sequences corresponding to bases 108-121, 139-154, 157-167, 365-378, 497-511, 598-613, 1123-1140, 1144-1161, 1285-1297, 1750-1762, 2001-2023, 2365-2378, 2931-2944 of SEQ. ID No. 1, including allelic, species and other sequence variants thereof, and

said second non-coding sequence is selected from the group of DNA sequences corresponding to bases 491-503, 737-747, 891-903, 994-1006 of SEQ. ID No. 1, including allelic, species and other variants thereof.

17. A method for identifying a candidate compound for the ability to modulate expression of OP-1, said method comprising the steps of:

- (a) incubating said candidate compound with a cell according to claim 11 or 13;
- (b) measuring the level of reporter gene expressed in said cell; and
- (c) comparing said level with that of said reporter gene expressed in said cell in the absence of said candidate compound, wherein a change in reporter



gene expression level is indicative of said candidate compound's ability to modulate OP-1 expression.

18. A kit for identifying a candidate compound capable of modulating OP-1 expression in a cell, the kit comprising:

- (a) a cell comprising transfected DNA sequences encoding;
  - (i) a reporter gene in operative association with at least two OP-1-specific non-coding sequences which can modulate expression of said reporter gene, and
  - (ii) at least two Pax gene expression products;
- (b) means for detecting modulation of said reporter gene following exposure of said cell to said candidate compound.

19. The kit of claim 18 wherein said reporter gene comprises a DNA sequence encoding OP-1.

20. The cell of claim 10 wherein the reporter gene comprises a DNA sequence encoding OP-1.

DATED this 12<sup>th</sup> Day of December 2001

Creative Biomolecules, Inc

By their Patent Attorneys

CULLEN & CO



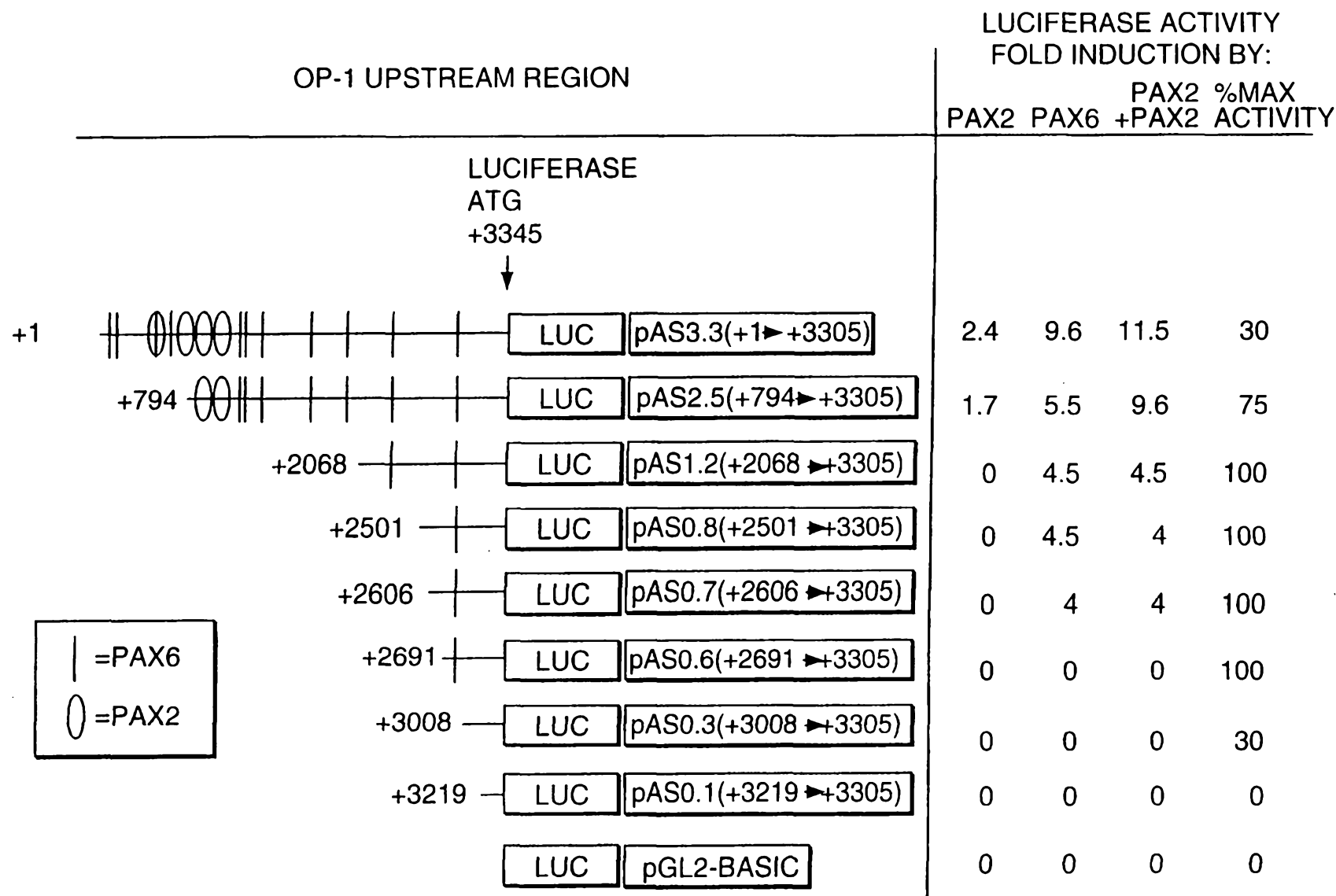


FIG. 1A

FIG. 1B

FIG. 2

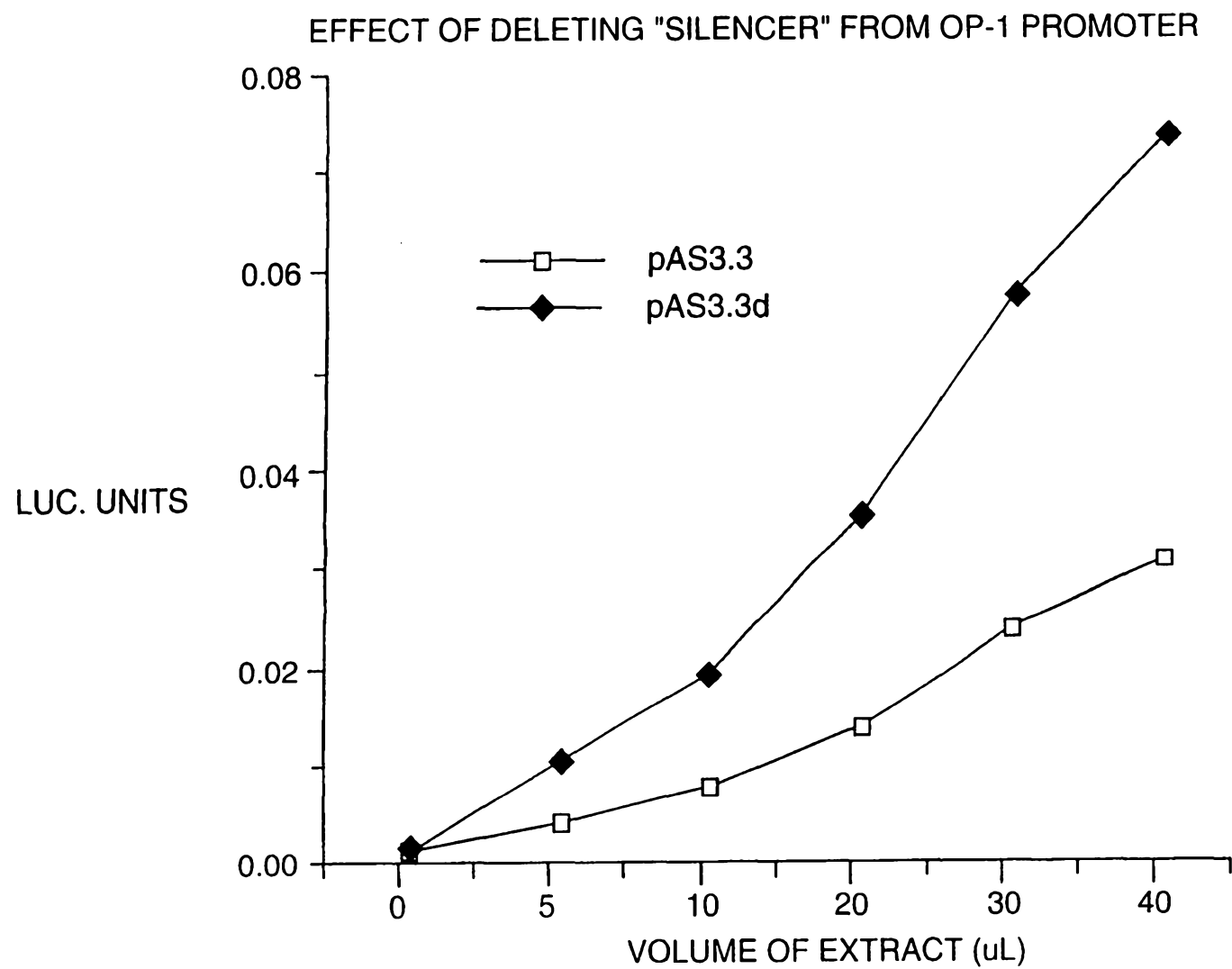


FIG. 3