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[54] **VOLTAGE-GATED CALCIUM CHANNEL AND ANTISENSE OLIGONUCLEOTIDES THERETO**

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[51] **Int. Cl.^{6}** **C12N 5/10**; C07H 21/00; C12Q 1/68

[52] **U.S. Cl.** **435/375**; 435/6; 536/23.5; 536/24.3; 536/24.31; 536/24.33; 536/24.5

[58] **Field of Search** 536/23.5, 24.3, 536/24.31, 24.33, 24.5; 514/44; 435/6, 240.2, 375; 935/33, 34, 36, 8, 9, 11

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	7/1983	Szoka, Jr. et al.	435/172
4,397,355	8/1983	McLamore	166/297
5,225,326	7/1993	Bresser et al.	435/6
5,585,479	12/1996	Hoke et al.	536/24.5

OTHER PUBLICATIONS

E. Uhlmann et al. Chemical Reviews 90 (4) (Jun. 1990) 543–84.

W. James Antivira Chem. & Chemotherapy 2(4) ('91) 191–214.

J. Holt et al. Mol. Cell. Biol. 8(2) (Feb. 1988) 963–73.

D. Tidd Anticancer Res. 10 ('90) 1169–82.

M. Cooney et al. Science 241 (Jul. 22, 1988) 456–9.

J. Milligan et al. J. Med. Chem. (Jul. 9, 1993) 36(14):1923–37.

C. Stein et al. Science 261 (Aug. 20, 1993) 1004–12.

B. Tseng et al. Cancer Gene Therapy 1(1) (Mar. 1994) 65–71.

W–J. Ma et al J. Biol. Chem. 267(32) (Nov. 15, 1992) 22728–32.

J. Wetmur Crit. Rev. Biochem. & Mol. Biol. 26 (3/4) ('91) 227–59.

Yamaguchi, D.T. et al.,*Journal of Biological Chemistry* 262: 7711–7718, "Parathyroid Hormone–activated Calcium Channels in an Osteoblast–like Clonal Osteosarcoma Cell" (Jun. 5, 1987).

Tanabe, T. et al. *Nature* 328, 313–318 "Primary Structure of the Receptor for Calcium Channel Blockers From Skeletal Muscle" (Jul. 23, 1987).

Yamaguchi, D.T. et al. *Journal of Biological Chemistry* 262: 14967–14973 "Protein Kinase C–activated Calcium Channel in the Osteo–blast–like Clonal Osteosarcoma Cell Line UMR–106*" (Nov. 5, 1987).

Ascenzi, A., *Journal of Biomechanical Engineering*, 110: 357–363 "The Micromechanics Versus the Macromechanics of Cortical Bone–A Comprehensive Presentation" (Nov. 1988).

Badley, J.E., et al., *Biotechniques* 6, 114–116 "A Simple Rapid Method for the Purification of Poly A+RNA" (1988).

Yamaguchi, D.T. et al.,*Journal of Biological Chemistry* 264: 4383–4390 "Characterization of Volume–sensitive, Calcium–permeating Pathways in the Osteosarcoma Cell Line UMR–106–01*" (Mar. 15, 1989).

Mikami, A. et al. *Nature* 340, 230–233 "Primary Structure and Functional Expression of the Cardiac Dihydropyridine–sensitive Calcium Channel" (Jul. 20, 1989).

Rothenberg, M., et al., *Journal of National Cancer Inst.*, 81:1539–1544, "Oligodeoxynucleotides as Anti–Sense Inhibitors of Gene Expression: Therapeutic Implications" (Oct. 18, 1989).

Koch, W. J. et al., *Journal of Biological Chemistry* 265, 17786–17791 "cDNA Cloning of Dihydropyridine–sensitive Calcium Channel from Rat Aorta" Oct. 15, 1990.

Perez–Reyes, E., et al.,*Journal of Biological Chemistry* 265, 20430–20436 "Molecular Diversity of L–type Calcium Channels" Nov. 25, 1990.

Iyer, R.P. et al., *J. Org. Chem.* 55:4693–4698 "The Automated Synthesis of Sulfur–Containing Oligodeoxyribonucleotides Using 3H–1,2–Benzodithiol–3–one 1,1–Dioxide as a Sulfur–Transfer Reagent" (1990).

Goodchild, J., *Bioconjugate Chemistry*, 1:1650167 "Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties" (1990).

Hui, A. et al., *Neuron* 7, 35–44 "Molecular Cloning of Multiple Subtypes of a Novel Rat Brain Isoform of the α_1 Subunit of Voltage–Dependent Calcium Channel" (Jul. 1991).

Snutch, T.P. et al.,*Neuron* 7, 45–57 "Distinct Calcium channels Are Generated by Alternative Splicing and Are Differentially Expressed in the Mammalian CNS" (Jul. 1991).

Naruse, K. et al.,*American Physiological Society* C1037–C1044, "Involvement of Stretch–Activated Ion Channels in Ca^{2+} Mobilization to Mechanical Stretch in Endothelial Cells" (1993).

Barry, E. et al., *Biotechniques* 15: 1018–1020 "Introduction of Antisense Oligonucleotides into Cells by Permeabilization With Streptolysin O" (Dec. 1993).

Primary Examiner—Charles C. P. Rories
Attorney, Agent, or Firm—Senniger, Powers, Leavitt & Roedel

[57] **ABSTRACT**

An antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the SA-Cat channel gene DNA or mRNA is provided, together with pharmaceutical compositions containing and methods utilizing such antisense oligonucleotide.

11 Claims, 6 Drawing Sheets

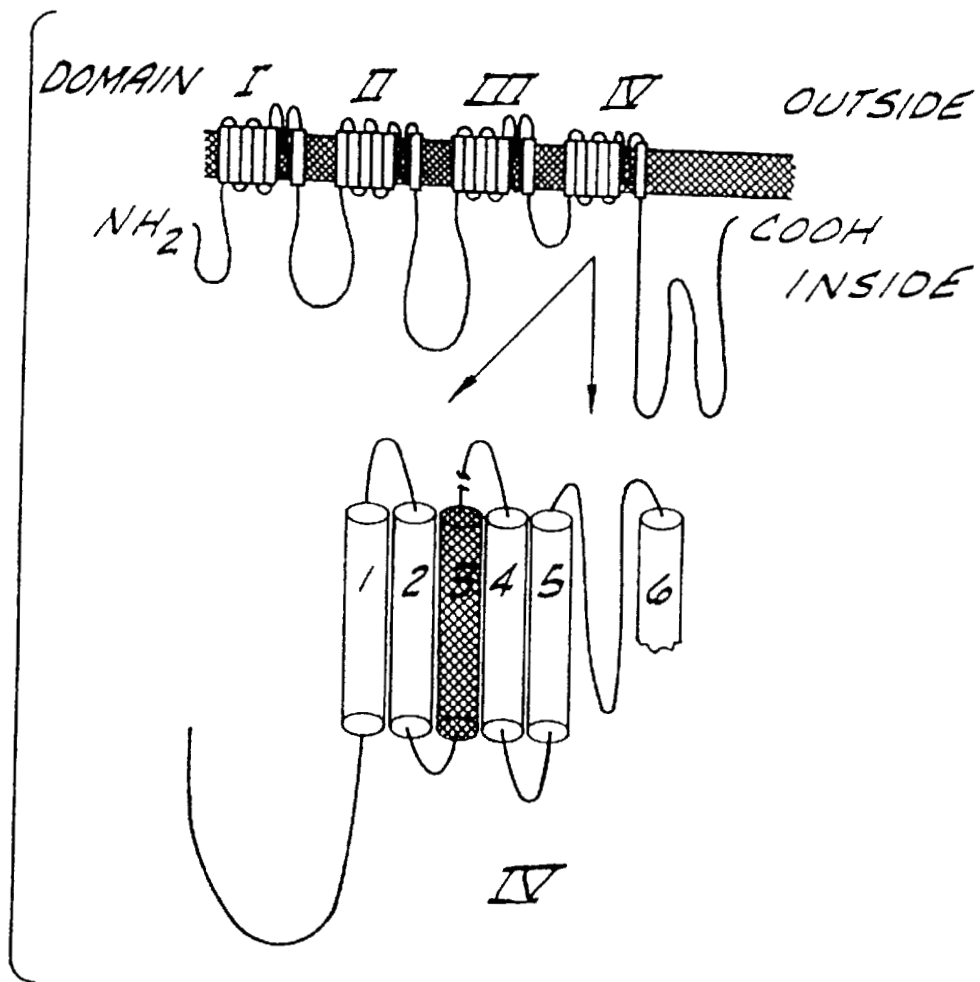# FIG. 1

FIG. 2

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ROB1        PWNVFDFLIVIGSIIDVILSEID........................DPDESARISSAFFRLFRVMRLIKLLSRA
(SEQ ID NO: 7)   | | | | | | | | | | | | | | | | |                | | | | | | | | | | | | | | | | | | | | | | | | | | |
RabSkel1 . PWNVFDFLIVIGSIIDVILSEIDTFLASSGGLYCLGGGCGNVDPDESARISSAFFRLFRVMRLIKLLSRA

ROB2        PWNVFDFLIVIGSIIDVILSETN.......................SAEENSRISITFFRLFRVMRLVKLLSRG
(SEQ ID NO: 9)   | | | | | | | | | | | | | | | | |                | | | | | | | | | | | | | | | | | | | | | | | | |
RatBr2     PWNVFDFLIVIGSIIDVILSETNPAEHTQCSPSM............SAEENSRISITFFRLFRVMRLVKLLSRG
(SEQ ID NO: 10)

ROB3        AWNTFDSLIVIGSIIDVALSEAD.......................NSEESNRISITFFRLFRVMRLVKLLSRG
(SEQ ID NO: 11)  | | | | | | | | | | | | | | | | |                | | | | | | | | | | | | | | | | | | | | | | | | |
RatBr-3    AWNTFDSLIVIGSIIDVALSEADPSDSENIPLPTATPG.....NSEESNRISITFFRLFRVMRLVKLLSRG
(SEQ ID NO: 12)
                          |_____IVS3_____|                |_____IVS4_____|
```
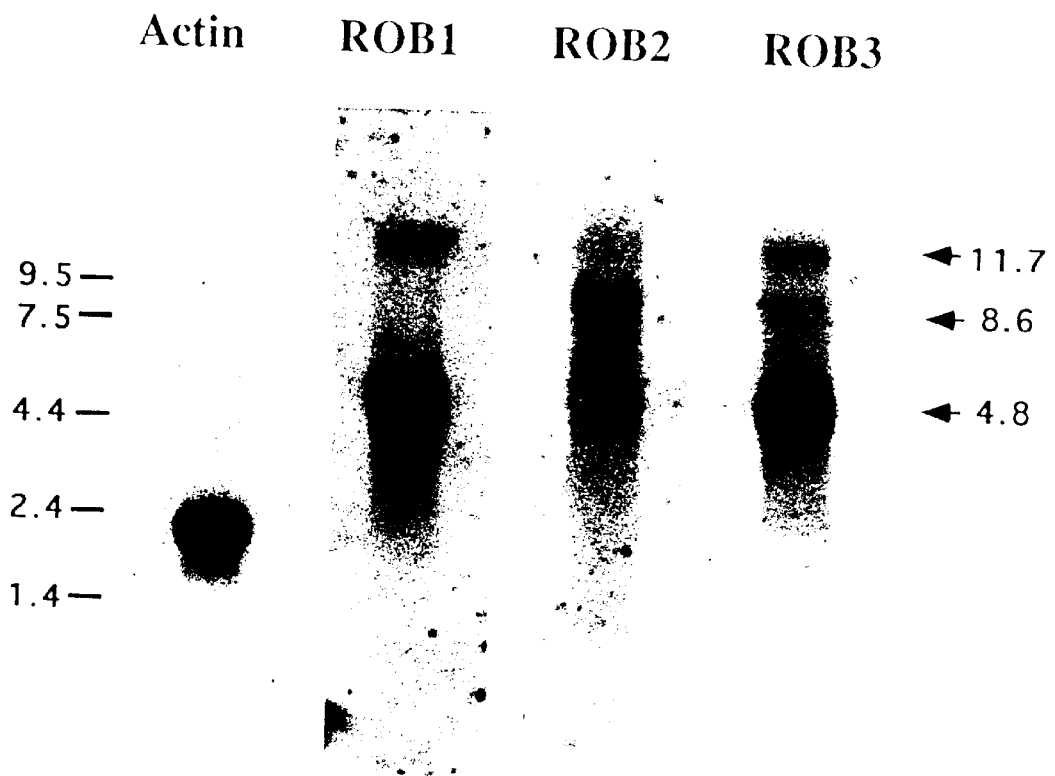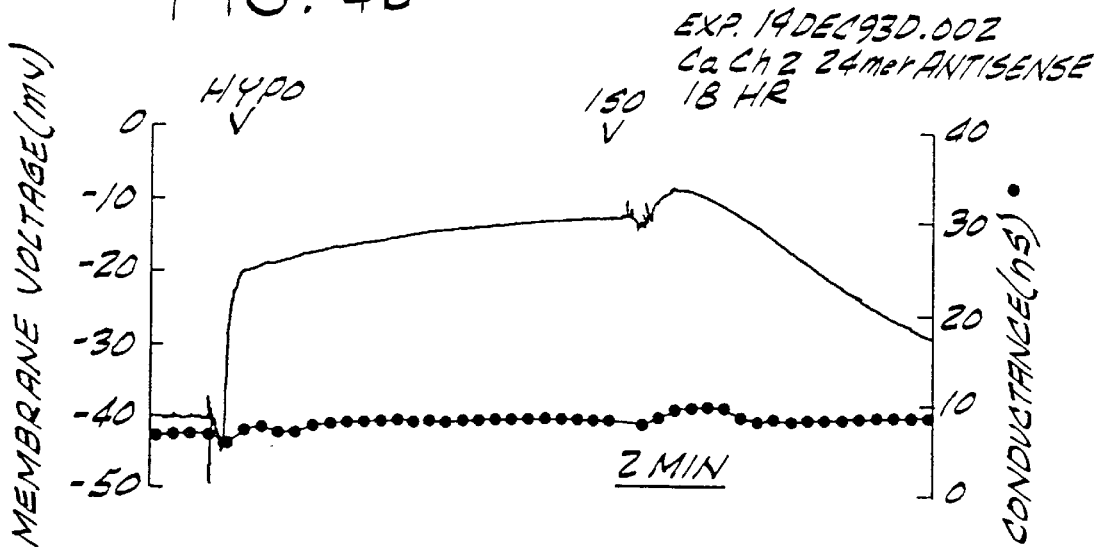
FIG. 3

FIG. 4a
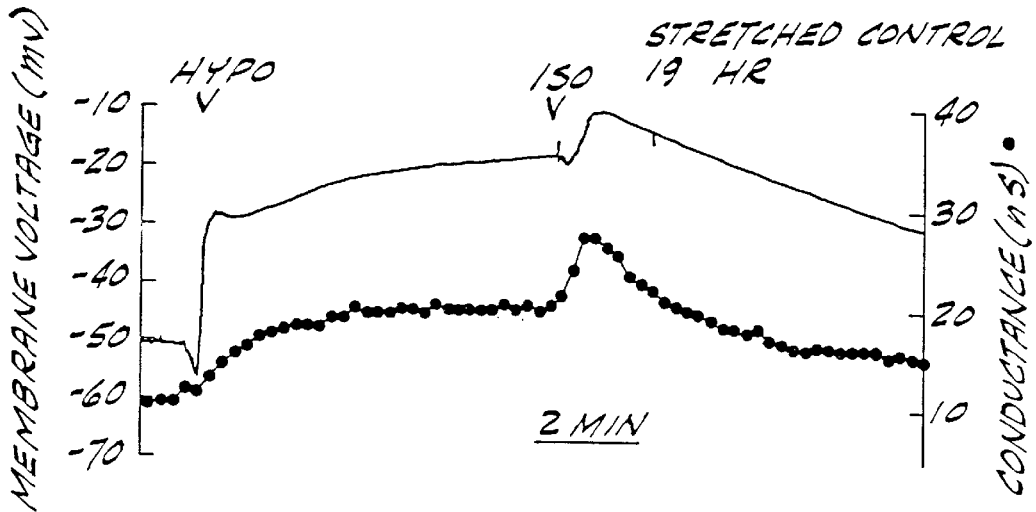
FIG. 4b

FIG. 5A
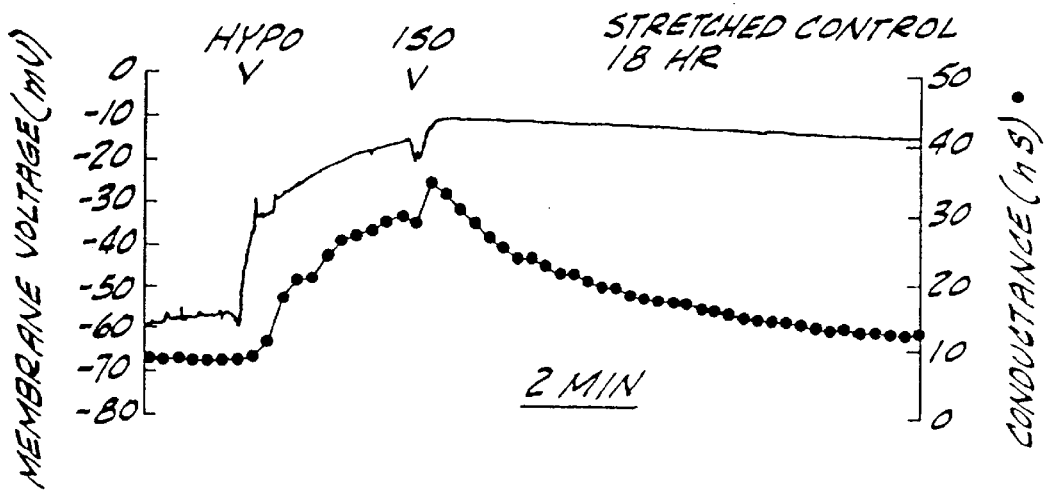
FIG. 5B
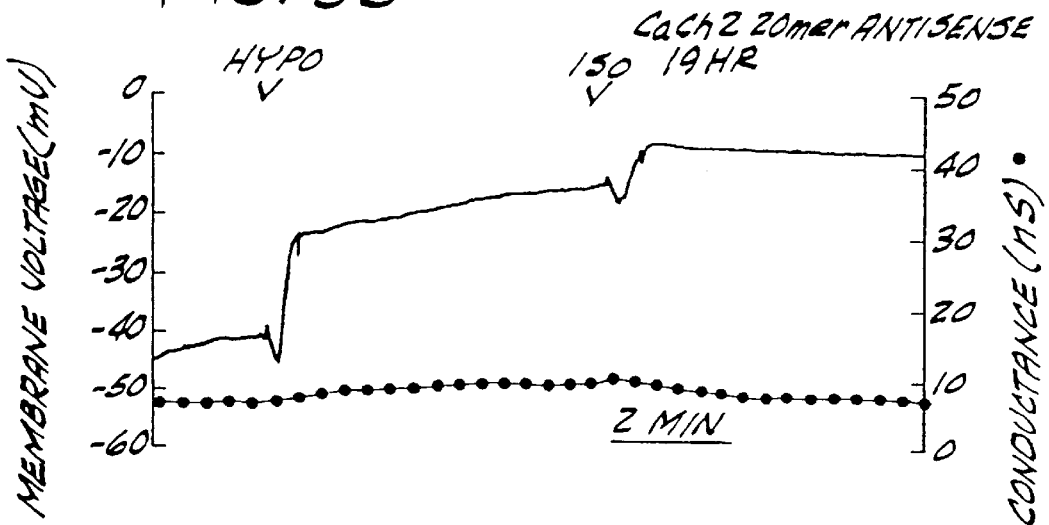
FIG. 6
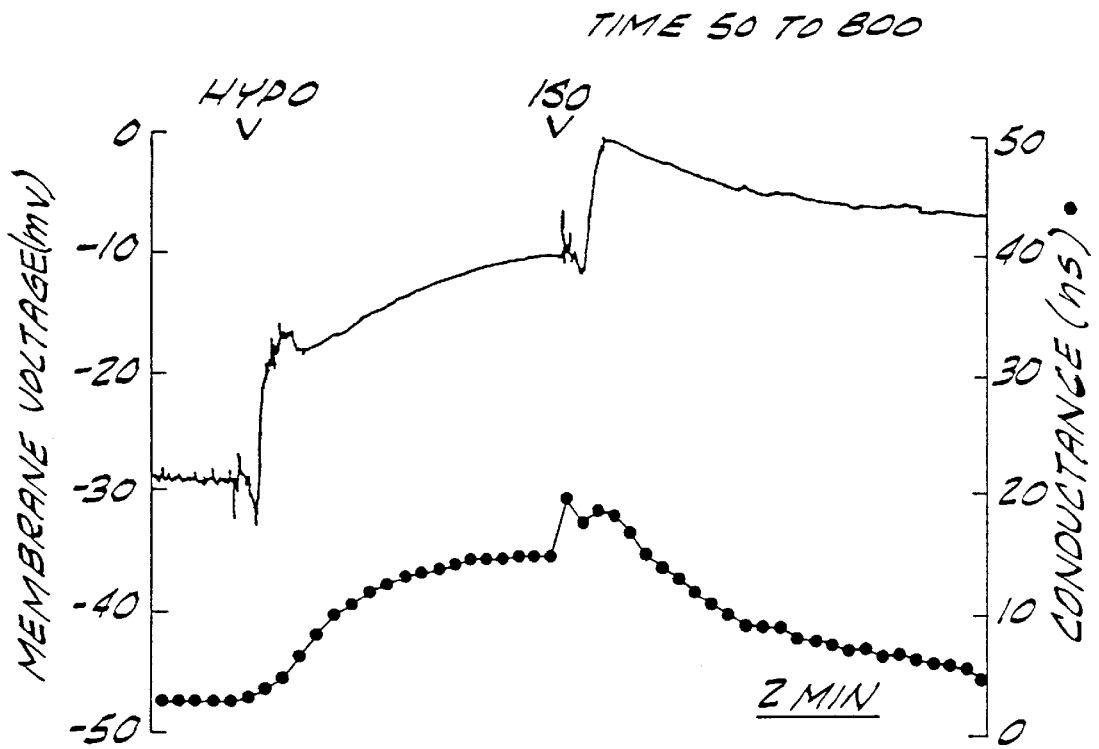
VOLTAGE-GATED CALCIUM CHANNEL AND ANTISENSE OLIGONUCLEOTIDES THERETO

BACKGROUND OF THE INVENTION

This invention relates to a voltage-gated calcium channel that stimulates bone formation in osteoblasts and mediates arterial tone in endothelial cells. More specifically, it relates to the gene for the voltage-gated calcium channel, antisense oligonucleotides useful for regulating the expression of the voltage-gated calcium channel gene and processes for the use of the antisense oligonucleotides to inhibit expression of the voltage-gated calcium channel, to inhibit bone formation, to mediate vascular blood pressure, and as an analytical tool useful for the development of strategies for responding to the bone loss caused by osteoporosis.

It has been known for a century that mechanical strain—whether resulting from physiological exercise or artificial means—increases bone formation and remodeling activity resulting in increased bone mass. Similarly, endothelial cells subjected to mechanical forces via fluid shear and vessel expansion have been shown to undergo increased cell proliferation and vasoactive secretions. Prior to the present invention, however, the precise molecular mechanism for enhancing the amount of bone deposited at the vector of force applied in osteoblasts and for increasing endothelial cell proliferation and vasoactive secretion, has remained unclear.

Voltage-gated calcium channels are members of a superfamily of cation channels, which also includes channels for Na^+, K^+ and divalent cations, i.e., Ca^{2+}. Calcium channels are responsible for the rapid entry of calcium into excitable cells upon membrane depolarization and are essential for excitation-contraction coupling in muscle, and the propagation of action potentials and neurotransmitter release in neurons. In addition, calcium channels regulate stimulus-secretion coupling during hormone release in endocrine glands. Much less is known about the roles of these calcium channels in other types of nonexcitable tissues.

Calcium channels may be sub-classified according to their electrophysiological behavior and pharmacological sensitivities. L-type calcium channels are defined pharmacologically by their sensitivity to three classes of calcium channel blockers, the phenylalkylamines, benzothiazepines and dihydropyridines. L-type calcium channels are multi-component proteins made up of five subunits: α_1, α_2, δ, γ, and β. The properties of calcium channels are largely conferred by the α_1 subunit, which forms the actual pore site for the channel. The structure of the α_1 subunit has been elucidated. It consists of four homologous repeats (I–IV) consisting of six membrane-spanning domains (S1–S6). The dihydropyridine receptor for the L-type channel is located on the carboxyl end of the α_1 unit of the protein just beyond the S6 domain of the IV region.

Although common features of the structure of calcium channels have been established, variations in this structure have also been identified. At least six classes of calcium channel α_1 subunit genes have now been identified, three of which are members of the dihydropyridine-sensitive L-type subfamily, including skeletal muscle (CaCh1), cardiac

muscle (CaCh2) and neuroendocrine (CaCh3) isoforms. Still greater diversity is generated by alternative splicing.

Osteoblasts, the cells responsible for bone growth, have voltage-gated calcium (SA-Cat) channels that are known to play a role in bone formation. Recent studies indicate that a similar voltage-gated calcium channel is present in endothelial cells. Stretch-activated cation channels are, as the name suggests, activated by mechanical stimuli, e.g., mechanical loading or osmotic change. SA-Cat channels are voltage-independent, gadolinium-inhibitable and cation non-selective. In the present patent application we identify the osteoblast voltage-gated calcium channel as a member of the calcium channel superfamily, namely a CaCh2 isoform.

Osteoporosis is a debilitating disease characterized by the loss of normal bone density with the thinning of bone tissue and the growth of small holes in the bones. Osteoporosis frequently causes chronic pain, especially in the lower back, increased frequency of broken bones, loss of body height, and eventual loss of mobility and other body function. Osteoporosis has been classified into two categories. Primary osteoporosis, which includes post-menopausal osteoporosis, and secondary osteoporosis, which includes immobilization or disuse osteoporosis, together afflict approximately 20 million Americans.

Osteosclerosis is a lesser-known condition characterized by an abnormal increase in the density of bone mass. The condition occurs in various diseases and is often linked to poor circulation in the bone tissue, infection or tumor formation.

Hypertension is a common, generally asymptomatic disease marked by high blood pressure persistently exceeding 140/90. Essential hypertension, also known as primary hypertension, has no single known cause, and is the most prevalent form of hypertension, having been diagnosed according to some estimates, in over 50 million Americans. Secondary hypertension is linked to disease of the kidneys, lungs, glands or vessels. For example, pulmonary hypertension is a condition of unusually high pressure within the blood vessels of the lungs.

Antisense oligonucleotides are short synthetic nucleotide sequences formulated to be complementary to a portion of a specific gene or mRNA. They function by hybridizing to complementary sequences, resulting in selective arrest of expression of the complementary gene or mRNA. In particular, the cytoplasmic location of mRNA provides a readily accessible target for antisense oligonucleotides entering the cell. In addition to the use of antisense oligonucleotides as therapeutic agents due to their ability to block expression of a specific target protein, they also provide a useful tool for exploring regulation of the expression of a gene of interest in vitro and in tissue culture (see Rothenberg, M., et al., *Natl. Cancer Inst.*, 81:1539–1544, 1989).

The development of new therapeutic strategies against, and the creation of new analytical tools for, a better understanding of diseases such as osteoporosis, osteosclerosis, and hypertension, are greatly desired. It is particularly desirable to provide such tools and therapies that are highly specific to a target gene and protein, which at the same time, because of their specificity, do not substantially affect other proteins or body functions. The provision of antisense oligonucleotides for use in designing therapies and diagnostic tools for diseases and physiological responses related to voltage-gated calcium channel mediated physiological activities satisfies a long-sought need for such therapies and tools.

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SUMMARY OF THE INVENTION

Among the objects of the invention, therefore, may be noted the provision of antisense oligonucleotides and pharmaceutical compositions that are capable of inhibiting the function of the voltage-gated calcium gene or mRNA and the provision of such oligonucleotides and compositions that serve as useful tools for exploring the regulation of gene expression in vitro and in tissue culture for the voltage-gated calcium. Also provided are a method for selectively blocking transcription or translation of the voltage-gated calcium channel gene and mRNA, for halting disease processes associated with the expression of that gene, and therapeutic procedures for the treatment of osteosclerosis and hypertension.

Briefly, therefore, the present invention is directed to an antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA.

In a further embodiment, the invention is directed to a pharmaceutical composition comprising at least one antisense oligonucleotide of 10 to 35 nucleotides in length that is capable of hybridizing with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA and a pharmaceutically acceptable carrier.

In another aspect of the present invention, a method of inhibiting expression of the voltage-gated calcium channel gene is provided. The method comprises the steps of obtaining an antisense oligonucleotide of 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, combining the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, thereby inhibiting expression of the voltage-gated calcium channel protein.

In yet another embodiment of the instant invention, a method of treating hypertension using the antisense oligonucleotides to the voltage-gated calcium channel gene is provided. The method includes the steps of obtaining a hypertensive-reductive amount of an antisense oligonucleotide 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the SA-Cat channel gene DNA or mRNA, combining the hypertensive-reductive amount of the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA of endothelial cells lining the blood vessels, inhibiting expression of the voltage-gated calcium channel protein and thereby causing a reduction in blood pressure.

In a further embodiment of the instant invention, a method of treating osteosclerosis using the antisense oligonucleotides to the voltage-gated calcium channel gene is provided. The method includes the steps of obtaining an osteosclerotic-reductive amount of an antisense oligonucleotide 10 to 35 nucleotides in length that can hybridize with a region of the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA, combining the osteosclerotic-reductive amount of the antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and contacting the pharmaceutically active mixture with the α_1 subunit of the voltage-gated calcium channel gene DNA or mRNA of osteoblasts in a target tissue, inhibiting expression of the voltage-gated

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calcium channel protein and thereby causing a reduction in bone density in the target tissue.

Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

SEQ ID NO.1 depicts the cDNA encoding for a region of the α_1 subunit of the voltage-gated calcium channel protein.

FIG. 1 is a schematic depiction of a model of an L-type calcium channel α_1 subunit such as the voltage-gated calcium channel of the invention. The channel is composed of four homologous domains (I–IV) that are modeled to contain six transmembrane regions (S1–S6), and a segment (H5 or SS1–SS2) between the S5 and S6 domains, which is thought to loop into the membrane and line the external entrance to the pore of the channel. The IVS3 region is shaded to indicate that alternative splicing can generate two variants of this region. The hatched line just downstream of the S3 region indicates the site where a segment is omitted in the bone transcripts as a result of alternative splicing.

FIG. 2 depicts a comparison of the deduced amino acid sequences of the UMR-106 clone with its corresponding CaCh isoform in the region where alternative splicing generates a deletion (ROB2 to the rat brain CaCh2 transcript (RatBr2).

FIG.3 is an autoradiogram of a Northern blot of RNA hybridized to UMR-106, calcium channel riboprobes. A riboprobe corresponding to clones ROB2 was hybridized to poly (A^+) RNA (10 μg/lane) isolated from UMR-106 cells. The actin riboprobe was hybridized to one-tenth as much RNA (1 μg/lane). Autoradiography was for 67 hours (ROBs) or 6 hours (actin). The actin riboprobe, which was used as a control, hybridized to a single mRNA band of approximately 2.0 kB. An RNA ladder (Gibco/BRL) was used for size markers (in Kilobases). FIGS. 4(a) and 4(b) are graphic representations of the effect of a preferred antisense 24mer oligonucleotide from the α_1 subunit of the voltage-gated calcium channel gene (SEQ ID NO.:2) on the response of membrane potential and whole cell conductance to hypotonic stretch. FIG. 4(a) depicts the UMR-106 cell membrane potential and whole cell conductance response to hypotonic stretch and reversal to isotonic media. The cells had been exposed to chronic cyclic strain for nineteen hours. FIG. 4(b), depicts the same information as in FIG. 6(a), except that the cells had been loaded with the antisense oligonucleotide (SEQ ID NO:2).

FIGS. 5(A) and 5(B) are graphic representations of the effect of a preferred antisense 20mer oligonucleotide from the α_1 subunit of the voltage-gated calcium channel gene (SEQ ID NO.:3) on the response to hypotonic stretch. FIG. 7(a) depicts the UMR-106 cells exposed to anti-streptolysin O but without exposure to the antisense oligonucleotide, exposed to 18 hrs of chronic cyclic strain prior to hypotonic stretch. FIG. 5(B) depicts the same procedure, but with exposure of the cells to the 20mer antisense oligonucleotide during the permeabilization.

FIG. 6 is a graphic representation of the effect of a sense 24mer oligonucleotide corresponding to the same sequence as in FIG. 4(b) on the response of UMR-106 cells to hypotonic stretch (as a control). Cells were loaded with the sense oligonucleotide in parallel with the antisense oligonucleotide used in FIG. 4(b) eighteen hours prior to study during which time the cells were exposed to chronic cyclic strain as described in Example 2 (Methods).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein means a molecule comprised of two or more deoxyribonucleotides, ribonucleotides, analogs or derivatives thereof.

"Oligonucleotide analog" or "oligonucleotide derivative," as those terms are used in conjunction with this invention, refer to moieties that function similarly to deoxyribonucleotides or ribonucleotides, but that have non-naturally occurring portions. Thus, oligonucleotide analogs may have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur-containing species that are known to be useful in this art. They may also contain altered base units or other modifications consistent with the function of oligonucleotides in conjunction with this invention.

In accordance with the invention, it has been demonstrated that an isoform of the voltage-gated CaCh2b channel, an voltage-gated calcium channel protein, is activated by chronic mechanical loading and parathyroid hormone (PTH), two factors known to have anabolic or positive effects on bone formation in osteoblasts. Based on the ability of antisense oligonucleotides to the voltage-gated calcium channel protein to block the increase in whole cell conductance shown to be triggered by the application of chronic, intermittent mechanical strain to osteoblasts, it has further been demonstrated in accordance with this invention that the voltage-gated calcium channel is the signaling mechanism at the molecular level for increasing bone formation in response to mechanical strain.

As a consequence of these and other discoveries described herein, it is believed that application of an antisense strategy against expression of the voltage-gated calcium channel protein in osteoblasts will directly inhibit the abnormally-high bone density formation reflected in the condition known as osteosclerosis. Moreover, these discoveries provide an important analytical tool for and a critical link in the development of methods for the pharmacologic modulation of this channel for clinical treatment of osteoporosis. Thus, it is believed that stimulation of the voltage-gated calcium channel, which serves as a signal transducer for mechanical strain, for example by application of an agonist active in stimulating the expression of the voltage-gated calcium channel protein, will promote the beneficial properties of exercise on bone without the physical exertion that is difficult or impossible for elderly or paralyzed patients or for those unable to enjoy the beneficial effects of strain imposed by Earth's gravity due to extended space flight.

Recent laboratory research also has demonstrated that shear stress, or the mechanical stimulation of the endothelial cells lining the blood vessels by the flow of blood, plays a role in the control of blood pressure. Furthermore, stretching endothelial cells in vitro increases endothelin production and cell proliferation through an increase in intracellular calcium. The increase in intracellular calcium is blocked by gadolinium, a SA-Cat channel inhibitor, suggesting that the SA-Cat channel also transduces the mechanical strain induced by shear stress into cellular biochemical message either to relax or contract the blood vessel. Therefore, modulation of this channel by application of an antisense strategy is also believed to provide the clinician with a means of controlling blood pressure.

These discoveries and their application will be explained in greater detail below.

Mechanical strain increases bone formation and remodeling activity resulting in increased bone mass. Osteoblasts have stretch activated cation (SA-Cat) channels that are

modulated by parathyroid hormone. Chronic, intermittent strain increases the sensitivity of SA-Cat channels to stretch increasing the number of channels open at any given time period and increasing single channel conductance. As a result, an increase in whole cell conductance is demonstrable. Furthermore, spontaneous activity of these channels is observed in chronically strained osteoblasts. These properties have not been previously demonstrated for SA-Cat channels, and they demonstrate that during mechanical loading of the osteoblast, SA-Cat channels are an integral component of the electrical environment and ion flux of the cell.

Chronic, intermittent strain applied to osteoblast-like cells not only affects ion conductance through SA-Cat channels, it also increases gene transcription for the bone matrix proteins. Application of cyclic strain for 24–72 hrs to osteoblast-like cell cultures increases type-1 procollagen message and type-1 collagen secretion. Chronic, intermittent strain also upregulates osteopontin message levels and osteocalcin secretion independent of, but additive to, 1,25 $(OH)_2$ Vitamin D_3 stimulation.

Calcium channel transcripts in UMR-106 cells were identified using the homology-based reverse transcriptase polymerase chain reaction. Primers were designed from regions that are highly conserved between L-type calcium channels to amplify essentially the entire fourth domain. An 850 base pair clone was isolated, which, upon sequencing, was found to be a CaCh2b variant. In addition, clones corresponding to CaCh1b and CaCh3b variants were isolated. The nucleotide sequence of the cDNA for the CaCh2b clone is set forth in Table 1. This Table depicts the nucleotide sequence of a partial cDNA clone encoding the fourth domain of the α_1 subunit of L-type calcium channel isoforms CaCh1 (ROB1), CaCh2 (ROB2), and CaCh3 (ROB3) isolated from rat osteosarcoma UMR-106 cells. The putative transmembrane domains S1–S6 are indicated by heavy lines under the sequence. The symbol Δ indicates the site of omission of a segment by alternative splicing in the CaCh2b isoform.

Employing an antisense strategy, in accordance with the invention we have demonstrated the ability to inhibit the response of osteoblast-like cells to chronic, cyclic strain. Thus, we have shown that antisense oligonucleotides to the CaCh2 transcript eliminates the biophysical properties of the voltage-gated calcium channel in the cells. Voltage-gated calcium channel activity is eliminated by application of the antisense oligonucleotide.

Antisense therapy is the administration of exogenous oligonucleotides that bind to a target polynucleotide located within the cells. The term "antisense" refers to the fact that such oligonucleotides are complementary to their intracellular targets, e.g., the voltage-gated calcium channel gene or mRNA. See for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression,* CRC Press, 1989; and *Synthesis* 1:1–5 (1988). The voltage-gated calcium channel antisense oligonucleotides of the present invention may be RNA or DNA that is complementary to and stably hybridizes with the DNA of, or the mRNA derived from, the voltage-gated calcium gene. Preferably, the antisense oligonucleotides are designed to maximize their specificity for the CaCh2 isoform relative to the CaCh1 and CaCh3 isoforms. Such specificity may be achieved by selecting sequences that

TABLE 1

											100
(SEQ ID NO: 4)	RO81	GGGGGAGACT	GAGTATAAGA	ACTGTGAGCT	GGACAAGAAC	CAGCGCCAAT	GTGTGCAGTA	TGCCCTGAAC	GCCCGCCCGG	TGAGGTGTTA	CATCCCCAAA
(SEQ ID NO: 5)	RO82	GGGGGAACAA	GAATACAAGA	ACTGTGAGCT	GGACAAGAAC	CAGAGACAAT	GTGTGGAATA	TGCCCTCAAG	GCCCGACCCT	TGGCGAAGGTA	CATCCCCAAG
(SEQ ID NO: 6)	RO83	GGGAGAAAAG	GAGTATAAGA	ACTGTGAGCT	GGACAAAAAT	CAGCGTCAGT	GTGTGGAATA	TGCCTTGAAG	GCCCGCCCCT	TAAGGAGATA	CATCCCCAAA

		101									200
(SEQ ID NO: 4)	RO81	AACCCATACC	AGTATCAGGT	GTGGTATGTC	GTCACTTCCT	CCTACTTTGA	ATACCTGATG	TTTGCTCTCA	TCATGCTCAA	CACTATCTGC	CTAGGCATGC
(SEQ ID NO: 5)	RO82	AACCAGCACC	AGTACAAAGT	GTGGTACGTG	GTCAACTCCA	CCTACTTCGA	GTATCTGATG	TTCGTCCTCA	TCCTGCTCAA	CACCATCTGC	CTGGCCATGC
(SEQ ID NO: 6)	RO83	AACCCATACC	AGTACAAGTT	CTGGTACGTG	GTGAACTCCT	CGCCTTTCGA	ATATATGATG	TTTGTGCTCA	TCATGCTCAA	CACGCTCTGC	CTGGCCATGC

IVS1

		201									300
(SEQ ID NO: 4)	RO81	AGCATTACAA	CCAGTCGGAA	CAGATGAACC	ACATCTCGGA	CATCCTCAAT	GTGGCTTTCA	CCATCATCTT	CACCCTGGAG	ATGATCCTCA	AGCTCATAGC
(SEQ ID NO: 5)	RO82	AGCACTACGG	CCAGAGCTGC	CTCTTCAAAA	TCGCCATGAA	TATACTTTCA	ATGCTTTTCA	CTGGCCTCTT	CACGGTGGGAG	ATGATCCTCA	AGCTCATTGC
(SEQ ID NO: 6)	RO83	AGCACTATGA	GCAATCCAAG	ACGCCATGGA	ATGTTCAATG	CATTCTGAAC	ATGGTCTTCA	CGGGGGTCTT	CACCGGTTGAG	ATGGTTTTGA	AAGTCATCGC

IVS2

		301									400
(SEQ ID NO: 4)	RO81	TTTCAAGCCC	AGGGGCTATT	TTGGAGACCC	CTGGAACGTG	TTTGACTTTC	TAATCGTCAT	TGGCAGCATC	ATTGACGTTA	TCCTGAGTGA	GATGGATGAC
(SEQ ID NO: 5)	RO82	CTTCAAACCC	AAGGGTTACT	TTAGTGATCC	CTGGAATGTT	TTTGACTTCC	TCATCGTCAT	TGGGAGCATA	ATTGATGTCA	TTCTCAGTGA	AACTAATAGT
(SEQ ID NO: 6)	RO83	ATTTAAGCCC	AAGGGCTATT	TTAGTGACGC	CTGGAACACG	TTTGACTCCC	TCATCGTAAT	CGGCAGCATT	ATAGACGTGG	CACTCAGCGA	AGCTGACAAC Δ

IVS3

		401									500
(SEQ ID NO: 4)	RO81	CCAGATGAGA	GCGCCGCGCAT	CTCCAGTGCC	TTCTTCCGCC	TGTTCCGAGT	CATGCGACTG	ATCAACCTGC	TGAGCCGGGC	CCAGGGTGTG	CGCACCCTGC
(SEQ ID NO: 5)	RO82	GCAGAGGAGA	ACTCCCGCAT	CTCCATCACC	TCTTCCGCC	TCTTCCGGGT	CATGCCGCTG	GTGAAGCTGC	TGAGCCGAGG	GGAAGGCATC	CGGACCCTG
(SEQ ID NO: 6)	RO83	TCTGAAGAGA	GCAATAGAAT	CTCCATCACC	TTTTTCCGTC	TTTTCCGAGT	GATGCGGGTTG	GTGAAGAGTTC	TCAGCAGAGG	GGAAGGCATC	CGGACTGTGG

IVS-4

		501									600
(SEQ ID NO: 4)	RO81	TCTGGACGTT	CACCAAGTCC	TTCCAGGGCC	GGCTTTGCTG	TGCCGTATGT	CTACGCTGTC	TCTTCTTCAT	ATCGGCATGC	ATCGGCATGC	AGATCTTCGG
(SEQ ID NO: 5)	RO82	TGTGGACCTT	CATCAAGTCC	TTCCAGGCCC	GGCCCTTTTG	TGCCCTATGT	CTATGCAGTG	TGTTCTTTAT	ATTGGGGATGC	ATTGGGGATGC	AGGTATTTGG
(SEQ ID NO: 6)	RO83	TATGGACCTT	CATTAAGTCC	TTCCAGGCAC	CGCCCTCCTC	TCCCATATGT	CTACGCCGTC	TCTTCTTCAT	ATTGGCATGC	ATTGGCATGC	AGATGTTTGG

IVS5

TABLE 1-continued

		601										700
(SEQ ID NO: 4)	RO81	GAAGATCGCC	ATGGTGGACG	GGACGCAAAT	AAACCGGAAC	AACAACTTCC	AGACTTTCCC	ACAAGCTGTC	CTGCTGCTCT	TCAGGTGTGC	CACAGGGGCA	
(SEQ ID NO: 5)	RO82	GAAGATTGCC	CTGAATGACA	CCACAGAGAT	CAATCGGAAC	AACAACTTCC	AGACGTTCCC	TCAGGCTGTG	CTACTTGCTCT	TCAGGTGCGC	CACTGGGGAC	
(SEQ ID NO: 6)	RO83	GAAGGTTGCC	ATGAGAGATA	ACAACCAGAT	CAATAGGAAC	AACAACTTCC	AGACGTTTCC	CCAGGCAGTG	CTGCTGCTCT	TCAGGTCTGC	AACAGGGGAG	

		701										800
(SEQ ID NO: 4)	RO81	GCCTGGCAGG	AGATCCTGCT	GGCCTGCAGC	TACGGGAAAC	GCTGCGACCC	GGAGTCTGA.	.....CTACG	CACCGGGGGA	GGAGTACGCG	TGTGGCACCA	
(SEQ ID NO: 5)	RO82	GCCTGGCAGG	ATATCATGCT	AGCCTGTATG	CCAGGCAAGA	AGTGTGCTCC	AGAGTCTGAG	CCCAGCAACA	GCACGGAAGG	GGAGACACCC	TGTGGCACCA	
(SEQ ID NO: 6)	RO83	GCCCGGCAGG	AGATCATGCT	CACCTGCCTC	CCTGGGAAGC	TGTGTGACCC	GGACTCAGA.	.....TTACA	ACCCAGGACA	GGAATATACT	TGTGGGAGCA	

		801					850
(SEQ ID NO: 4)	RO81	ACTTTGCCTA	CTACTACTTC	ATCAGCTTCT	ACATGCTCTG	CGCCTTCCTG	
(SEQ ID NO: 5)	RO82	GTTTCGCTGT	CTTCTACTTC	ATCAGCTTCT	ACATGCTCTG	TGCCTTCCTG	
(SEQ ID NO: 6)	RO83	ACTTTGCCAT	TGTCTACTTC	ATCAGCTTTT	ACATGCTCTG	CGCGGTTCCTG	

IVS6

are not highly conserved between the isoforms. In general, only one or two (e.g., 1 in 20) base pair mismatches in a relatively short oligonucleotide are sufficient to exclude hybridization to non-targeted isoforms. The voltage-gated calcium channel antisense oligonucleotides of the present invention also include derivatives such as S-oligonucleotides (phosphorothioate derivatives or S-oligos) which exhibit voltage-gated calcium channel inhibitory action.

S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligos) in which a non-bridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention may be prepared by treatment of the corresponding O-oligos with 3H-1,2-benzodithiol-3-one-1,1-dioxide, which is a sulfur transfer reagent. See Iyer, R. P. et al., *J. Org. Chem.* 55:4693–4698 (1990), the disclosures of which are fully incorporated by reference herein. The above substitutions are known in the art to function to enhance the ability of the compositions to penetrate into the region of cells where the RNA whose activity is to be modulated is located. Others, such as alkyl phosphorothioate bonds, N-alkyl phosphoramidates, phosphorodithioates, alkyl phosphonates, and short chain alkyl or cycloalkyl structures may also be useful. Persons with ordinary skill in the art will be able to select other linkages for use in the practice of the invention.

Oligonucleotide analogs may also include species that include at least some modified base forms. Thus, purines and pyrimidines other than those normally found in nature may be so employed. Similarly, modifications on the furanose portions of the nucleotide subunits may also occur as long as the essential tenets of this invention are adhered to. All such analogs are comprehended by this invention so long as they function effectively to hybridize with DNA of or with mRNA derived from the voltage-gated calcium channel gene to inhibit the function of that DNA or mRNA.

The antisense oligonucleotides of this invention preferably comprise about 10 to about 35 bases, preferably from about 15 to 30, and most preferably about 18 to about 26. Two preferred embodiments of the antisense oligonucleotide sequences of the present invention are a 24 mer antisense oligonucleotide having SEQ. ID NO: 2 and a 20 mer antisense oligonucleotide having SEQ. ID NO: 3. These antisense oligonucleotides act just on the 5'-side of the S6 region of the IV domain of the voltage-gated calcium channel α_1 subunit.

The oligonucleotides of this invention are designed to hybridize with DNA or mRNA of the voltage-gated calcium channel. Such hybridization, when accomplished, interferes with the normal function of these DNA/mRNA components. Such inhibited functions may include transcription of the DNA to mRNA, translocation of the RNA to the site of protein translation, and actual translation of protein from the mRNA. The overall effect of the interference caused by the antisense oligonucleotides of the invention is to cause inhibition of the expression of the voltage-gated calcium channel protein. Since it has been established that the voltage-gated calcium channel protein is a component of the signaling mechanism for stretch-induced increased bone formation in osteoblasts, such inhibition works directly to reduce the abnormally-high bone density exhibited in patients suffering from osteosclerosis. Similarly, it is believed that inhibition of voltage-gated calcium channel expression in endothelial tissue by antisense oligonucle-otides will inhibit the enhanced cell proliferation and con-comitant cellular changes which cause hypertension.

Although use of the antisense strategy is not directly applicable to therapies for treating osteoporosis, antisense oligonucleotides of the present invention represent impor-tant analytical tools for studying mechanisms to trigger, rather than inhibit, the expression of voltage-gated calcium channels in osteoblasts to signal enhanced bone matrix formation. Such further investigation using the antisense oligonucleotides as an analytical tool may provide the basis for developing agonists for the voltage-gated calcium chan-nel signaling mechanism that can upregulate the level of bone matrix formation to counteract the loss of bone density caused by osteoporosis.

The present invention is additionally useful in diagnostics and in research in respects other than those specifically referenced above. It is believed that the voltage-gated cal-cium channels play roles in tissues not yet fully explored. Since the oligonucleotides of this invention hybridize to voltage-gated calcium channels, sandwich and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotides with voltage-gated calcium channels in tissue samples suspected of containing this channel may be accomplished by such means as enzyme conjugation, radiolabelling or other suit-able detection systems.

Also included in the present invention are pharmaceutical compositions comprising an effective amount of at least one of the voltage-gated calcium channel antisense oligonucle-otides of the invention in combination with a pharmaceuti-cally acceptable carrier. The voltage-gated calcium channel antisense oligonucleotides are preferably coadministered with an agent which enhances the uptake of the antisense molecule by the cells. For example, the voltage-gated cal-cium channel antisense oligonucleotides may be combined with a lipophilic cationic compound, which may be in the form of liposomes. The use of liposomes to introduce nucleotides into cells is taught, for example, in U.S. Pat. Nos. 4,397,355 and 4,394,448, incorporated herein by ref-erence. Alternatively, the voltage-gated calcium channel antisense oligonucleotides may be combined with a lipo-philic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A pre-ferred sterol is cholesterol.

In addition, the voltage-gated calcium channel antisense oligonucleotide may be conjugated to a peptide that is ingested by cells. Examples of useful peptides include peptide hormones or antibodies. By choosing a peptide that is selectively taken up by the neoplastic cells, specific delivery of the antisense agent may be effected. The voltage-gated calcium channel antisense oligonucleotide may be covalently bound via the 5'H group by formation of an activated aminoalkyl derivative. The peptide of choice may then be covalently attached to the activated voltage-gated calcium channel antisense oligonucleotide via an amino and sulfydryl reactive hetero bifunctional reagent. The latter is bound to a cysteine residue present in the peptide. Upon exposure of cells to the SA-Cat channel antisense oligo-nucleotide bound to the peptide, the peptidyl antisense agent is endocytosed and the voltage-gated calcium channel anti-sense oligonucleotide binds to the target voltage-gated cal-cium channel mRNA to inhibit translation.

The voltage-gated calcium channel antisense oligonucle-otides and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, or transdermal routes. The dosage adminis-tered will be dependent upon the age, health, and weight of

the recipient, type of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For treatment of osteoscierosis, an osteosclerotic-reductive amount is to be administered. For treatment of hypertension, a hypertensive-reductive amount is to be applied.

Compositions within the scope of this invention include all compositions wherein the voltage-gated calcium channel antisense oligonucleotide is contained in an amount that is effective to achieve inhibition of expression of the voltage-gated calcium channel protein sufficient to, when applied to osteoblasts, ameliorate osteosclerosis, and when applied to endothelial tissue, cause a measurably significant reduction in blood pressure, either in localized tissue or system-wide. Although individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art.

In addition to administering the voltage-gated calcium channel antisense oligonucleotides as a raw chemical in solution, the voltage-gated calcium channel antisense oligonucleotides may be administered as part of a pharmaceutically active mixture or preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the voltage-gated calcium channel antisense oligonucleotide into preparations that can be used pharmaceutically.

Suitable formulations for parenteral administration include aqueous solutions of the voltage-gated calcium channel antisense oligonucleotides in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The antisense oligonucleotides of the present invention may be prepared according to any of the methods that are well known to those of ordinary skill in the art. Preferably, the antisense oligonucleotides are prepared by solid phase synthesis. See, Goodchild J. *Bioconjugate Chemistry*, 1:1650167 (1990), for a review of the chemical synthesis of oligonucleotides. Alternatively, the antisense oligonucleotides can be obtained from a number of companies which specialize in the custom synthesis of oligonucleotides.

The following examples illustrate the invention.

EXAMPLE 1

Materials—UMR-106 cells were obtained from American Type Culture Collection (Rockville, Md.). Cell culture media and serum were purchased from Sigma. Sources for other materials were as follows: Fast Track mRNA Isolation Kit (Invitrogen Corp., San Diego, Calif.), GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, Conn.), pBluescript KS⁻ vector and XL-1 Blue bacteria (Stratagene, La Jolla, Calif.), Nuseive, Seakem and Seaplaque agarose (FMC Bioproducts, Rockland, Me.), Sequenase II kit (United States Biochemical Corp., Cleveland, Ohio), DNA ligase (Boehringer Mannheim, Indianapolis, Ind.), acrylamide (Gel-Mix 8, Gibco/BRL, Gaithersburg, Md.), DNA purification kit PZ523 Kit (5 Prime→3 Prime, Inc., Boulder, Colo.), Genescreen Plus nylon membrane (Du Pont NEN Research Products, Boston, Mass.). Labeled probes were prepared using the Riboprobe Gemini System (Promega Corp., Madison, Wis.). [α³²P]UTP was purchased from Amersham. Oligonucleotides were synthesized commercially by Macromolecular Resources (Fort Collins, Colo.). Restriction enzymes were purchased from Gibco/BRL.

Cell Culture—UMR-106 cells were cultured in D-MEM/Ham's F-12 medium containing 5% fetal bovine serum. Cells were used between passages 3 and 17.

Purification of RNA—Poly (A⁺) RNA was isolated from cultured cells as described in Bradley, J. E., et al., *Biotechniques* 6, 114–116 (1988) using the FastTrack mRNA Isolation Kit. Briefly, UMR-106 cells were grown to confluence in P-100 culture dishes, washed in phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and harvested by scraping. Cells were pelleted by centrifugation and homogenized with a polytron in the presence of 2% SDS. After incubation with proteinase K, the cell lysate was batch adsorbed to oligo (dT) cellulose and poly (A⁺) RNA was eluted in salt-free buffer. Approximately 20 μg of poly (A⁺) RNA was isolated from 1×10₈ cells.

cDNA synthesis and PCR—Poly (A⁺) RNA was reverse transcribed and cDNA was amplified using the GeneAmp RNA PCR kit as follows. Poly (A⁺) RNA, 250 ng, was incubated in a 20 μl reaction volume containing 2.5 U/μl Moloney murine leukemia viral reverse transcriptase, 2.5 μM random hexamers, 1.0 mM dNTPs and 1 U/μl RNase inhibitor in 50 mM KCl, 5 mM MgCl₂, and 10 mM Tris-HCl, pH 8.3, for 10 min at room temperature followed by 15 min at 42° C. and 5 min at 99° C. Subsequently, the samples containing first strand cDNA were brought up to a 100 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM MgCl₂, 200 μM of each dNTP, 0.2 μM of each primer, and 2.5 units of AmpliTaq DNA polymerase and overlaid with 75 μl of mineral oil. PCR was performed on an automated thermal cycler (Model 480, Perkin-Elmer Cetus) that was programmed to repeat the following cycle 30 times: 60 s at 94° C., 60 s at 60° C. and then 120+2 s/cycle at 72° C.

PCR primers—Oligodeoxynucleotide PCR primers were designed from regions that are highly conserved between L-type calcium channels using the following published sequences: CaCh 1 (rabbit skeletal muscle isoform) Tanabe, T. et al. (1987) *Nature* 328, 313–318, CaCh2 (rat cardiac muscle isoform) Snutch, T. P. et al. (1991) *Neuron* 7, 45–57 and CaCh3 (rat neuroendocrine isoform) Hui, A. et. al. (1991) *Neuron* 7, 35–44. Two pairs of primers were used. One set spanned the entire calcium channel domain IV: upstream primer-1 (UP-1): 5'-gcc ggatccatcgtcaccttccaggagca-3'(SEQ ID NO: 13); and downstream primer (DP-1): 5'-atggaattcgccacraagaggttgatgat-3' (r=a or g)(SEQ ID NO: 14). A second set spanned domain IVS5 through IVS6: upstream primer-2 (UP-2): 5'-gtg ggaattcatcaagtccttccaggccct-3'(SEQ ID NO: 15) and downstream primer-2 (DS-2): 5'-cagg ggatccaagttgtccatgataacagc-3'(SEQ ID NO: 16). An additional downstream primer, (DS-3): 5'-ccc gaattcakmgtgttgagcatgatgag-3' (k=t or g, m=a or c)(SEQ ID NO: 17), was designed from sequence in the IVS1 segment of the UMR-106 ROB1 and ROB3 clones to be used with the UP-1 primer to amplify only the 5' end of those clones. Bam H1 or Eco R1 restriction sites (underlined) were included near the 5' ends of the primers to facilitate directional subcloning.

Cloning PCR products—The products of the PCR reaction were directionally cloned into the pBluescript KS⁻ vector. Initially, one half of each PCR reaction was analyzed by electrophoresis on 1% Nuseive/1% Seakem agarose gels. For subcloning, the remaining reaction product was purified by phenol/chloroform/Isoamyl alcohol (25:24:1) extraction and ethanol precipitation, digested with Eco R1 and Bam H1, and isolated as a band from a low melting temperature

1% Seaplaque agarose gel. The vector was also digested with Eco R1 and Bam H1 and isolated on a low melt agarose gel. The ligation reaction was carried out in a 50 μl volume containing 5 U T4 DNA ligase in 66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithioerytritol, 1 mM ATP, pH 7.5. Agarose gel slices containing the PCR product or vector were melted at 70° C. for 10 min before the addition of 2–4 μl to the reaction. The ligation reaction was incubated at 15° C. for 16 h. One tenth of each ligation reaction was used to transform XL-1 Blue bacteria. Competent bacteria were prepared by the calcium chloride method and transformed according to a standard protocol (Sambrook, J., et al., *Molecular Cloning: A Laboratory Manual,* 2nd Ed., 1989). Transformants were plated onto LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) agar plates containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline that had been coated with 10 μl of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside and 40 μl of 100 mM isopropyl-β-D-thiogalactopyranoside. Individual white colonies were picked and grown overnight in 5 ml of LB medium containing 50 μg/ml ampicillin at 37° C. Plasmid DNA was prepared from 3.0 ml of overnight culture by alkaline lysis. The presence of an appropriately sized insert was confirmed by Eco R1 and Bam H1 restriction digestion of the plasmid DNA.

Sequencing of Double-Stranded DNA Templates— Plasmid DNA, 5 μg, was denatured in 200 mM NaOH, 0.2 mM EDTA for 30 min at 37° C., neutralized and precipitated in ethanol. Sequencing was by the dideoxynucleotide chain termination method using the Sequenase II kit. The products were separated by electrophoresis on 8.0% acrylamide gels. Some sequencing was also performed on an automated instrument (Applied Biosystems, Foster City, Calif.) using plasmid DNA purified with the PZ523 Kit. The final sequences were determined from both strands of the cDNA. Sequence analysis was performed using on-line software from the Genetics Computer Group, Inc. (Madison, Wis.).

Northern Blot Analysis—Poly (A+) RNA was electrophoresed on a 0.75% agarose/formaldehyde denaturing gel and eletrophoretically transferred to Genescreen Plus nylon membrane. The membranes were UV-crosslinked and baked for 2 h at 80° C. under vacuum. Prehybridization was carried out in 0.25 m $NaPO_4$, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 50% formamide, 6% SDS at 55° C. for 1 hr. [^{32}P]-labeled RNA probes were synthesized form linearized cDNA templates using the Riboprobe Gemini System. Hybridization with riboprobes, 2×10^6 cpm/ml, was under the same conditions as the prehybridization for 20 h. The membranes were washed twice in 0.3M NaCl, 0.03M Na_3Citrate, pH 7.0 ($2\times$ SSC) for 5 min at room temperature; twice in 0.25M $NaPO_4$, pH 7.2, 2% SDS, 1 mM EDTA for 30 min at 65° C.; and twice in 0.04 M $NaPO_4$, pH 7.2, 1% SDS, 1 mM EDTA for 30 min at 65° C. To decrease the background, the membranes were subsequently rinsed three times in $2\times$ SSC for 5 min at room temperature, incubated in $2\times$ SSC containing 1 μg/ml RNase A for 15 min at room temperature, and washed twice in 0.04M $NaPO_4$, pH 7.2, 1% SDS, 1 mM EDTA for 30 min at 65° C. Autoradiography was carried out at −70° C. with an intensifying screen. Hybridization with a riboprobe for human β-actin was used as a positive control.

RESULTS

Homology-based reverse transcriptase-PCR was used to identify transcripts for L-type calcium channels that are present in UMR-106 cells. Primers were designed from regions that are highly conserved between L-type calcium channels to amplify essentially the entire fourth domain (FIG. **1**). Three types of cDNA clones were isolated, corresponding to all three of the known L-type calcium channel genes. The CaCh2 isoform (Table 1, ROB2), 850 base pairs, is identical to the rat brain rbC-I transcript (22), except for a 33 base pair (11 amino acids) deletion (FIG. **2**) in the S3–S4 linker. This isoform has subsequently been determined (see below) to be a close isoform of the voltage-gated calcium channel gene.

Alternative splicing in the fourth domain of L-type calcium channels can potentially give rise to four variants of CaCh2 (CaCh2a, b, c, d) transcripts (Perez-Reyes, E., et al., *J. Biol. Chem.* 265, 20430–20436 (1990)). This occurs through the use of an alternate splice acceptor site or an exon skipping event in the extracellular IVS3-S4 linker that produces two sizes of transcripts. In addition, mutually exclusive splicing of alternate exons (approximately 50% homologous) encoding the transmembrane IVS3 domain occurs in CaCh2 transcripts (Perez-Reyes, Supra).

According to the Perez-Reyes nomenclature, each of the three L-type CaCh transcripts isolated from UMR-106 cells are of the b type since they contain a specific IVS3 variant and they lack a portion of the IVS3-S4 linker region.

Northern analysis was used to detect expression of mRNA transcripts corresponding to the UMR-106 calcium channel clones (FIG. **3**). (Three sizes of mRNAs of approximately 11.7, 8.6, and 4.8 kB were detected.) The ROB2 clone hybridized predominately to the 8.6 kB MRNA, which is the expected size for a CaCh2 isoform transcript. However a larger 12–15 kB transcript detected in other tissues (22,26, 27), including cardiac muscle, aorta, and brain, was not seen. Very high stringency was used during Northern analysis to maximize the specificity of hybridization. Nevertheless, it is possible that the high degree of sequence identity (approximately 76%) between each of the three CaCh isoforms in the region cloned may be responsible for some cross-hybridization as a result of the relatively high abundance of those transcripts.

EXAMPLE 2

METHODS

Cell culture: UMR-106.01 cells (passages 12–18) were grown in Minimal Essential Medium with Eagles modification, non-essential amino acids and Earle's salts (Sigma, St. Louis, Mo.) supplemented with 10% fetal bovine serum (Gibco, Grand Island, N.Y.). Cells were plated onto flexible, type I collagen-coated, silicone-bottomed 6 well culture plates (Flexcell, Inc., McKeesport, Pa.), fed twice weekly and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37° C. When the cells were approx. 75% confluent, they were transfected with either the sense or antisense oligodeoxynucleotides (oligonucleotide) as described below. Culture plates were placed on the Flexercell apparatus which uses vacuum to stretch the silicone bottoms and cyclic stretch was applied for 12–30 hrs at 3 cycles/min. A maximal strain of 12% displacement was applied at the edge of the culture plate falling to 0% displacement at the center.

Transfection of oligonucleotide's. Two antisense oligonucleotides (24-mer and 20-mer)(respectively, SEQ ID NO: 2 and NO: 3) and a sense (24-mer) oligonucleotide were developed from the sequence of a cDNA clone of the α_1 subunit of an L-type calcium channel (CaCh2) isolated from UMR-106 by reverse transcriptase-polymerase chain reaction (SEQ ID NO:1). The antisense oligonucleotides were commercially synthesized by Macromolecular Resources (Fort Collins, Colo.). The antisense oligonucleotides were introduced into the UMR cells using streptolysin O (Sigma, St. Louis, Mo.) permeabilization. After the UMR cells had

been plated onto the flexible, silicone-bottomed culture plates and grown to 75% confluency, the medium was removed and the cells were washed with a permeabilization buffer consisting of (in mM): 137 NaCl, 5.6 glucose, 2.7 KCl, 2.7 EGTA, 1 Na-ATP, 100 PIPES and 0.1% bovine serum albumin, pH 7.4. The permeabilization buffer containing 0.5 U/ml strptolysin O and $100 \mu M$ of the appropriate oligonucleotide was then placed on the cells for 5 min at room temperature. This solution was then removed and the normal medium with 10% fetal bovine serum was added to the cells. For control experiments, UMR cells were permeabilized with the same concentration of streptolysin 0 with no oligonucleotide present.

Patch Clamp Studies. Following application of chronic, intermittent strain, the silicone bottom of the cluster was removed and transferred to a recording chamber (1 ml total volume (Biophysica Technologies, Baltimore, Md.) which was modified to permit rapid exchange of the bathing solution with minimal perturbation to the cells. Cells were bathed in a mammalian Na^+ Ringer's solution consisting of (in mM): 136 NaCl; 5.5 KCl; 1 $MgCl_2$; 1 $CaCl_2$; and 20 N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid] (HEPES) buffer; titrated to pH 7.3 with NaOH. The strain pattern associated with the Flexcell apparatus is non-uniform (1). The profiles of strain range from $120,000 \mu E$ at the edge of the well (12% maximal displacement) to $0 \mu E$ at the center. The cells used in this study were subjected to similar magnitudes of strain since patches were always performed in an area 10mm from the edge of the well. Strain applied to this area estimated at 10,000 to $20,000 \mu E$ using the strain curve described by Banes, et al. Comparisons were made between chronically stretched control and oligonucleotide-treated cells from the same passage number and at the same level of confluency. To impose membrane strain on the UMR cells during the patch clamp studies, 10 ml 65 mM NaCl (182 mOsm) hypotonic Ringer's solution was perfused into the chamber.

To measure membrane potentials and whole cell conductances, the nystatin performed patch technique was used under current clamp conditions. The pipette solution in these experiments consisted of (in mM): 12 NaCl; 64 KCl; 28 K_2SO_4; 47 sucrose; 1 $MgCl_2$; 0.5 EGTA; 20 HEPES; titrated to 7.35 with KOH. Nystatin was added at a concentration of 300 $\mu g/ml$ to permeabilize the patch. Access resistances of <40 MΩ were consistently achieved with this concentration of nystatin. Whole cell conductance measurements were made by pulsing ±50 pA across the membrane.

RESULTS

We examined the effects of the antisense and sense oligonucleotides on the strain-induced increase in whole cell conductance in UMR-106.01 cells, which we have shown to be the result of upregulation of the stretch activated cation channel. A previous report had demonstrated that antisense oligonucleotides produce a time dependent inhibition of the chlorothiazide-induced increase in $[CaU^{2+}]_i$ in the mouse distal convoluted tudbule cells. This inhibition starts 6 hrs after introduction of the antisense and peaks at 18 hrs. The delay in inhibition was attributed to the turnover of existing proteins prior to the inhibition of new synthesis by the antisense. Cell conductance measurements of the strained UMR cells which had been transfected with the 24-mer antisense found a similar time dependence which began at 12 hr. Measurements at 18 hr found 100% inhibition of the strain-induced increase in whole cell conductance (FIGS. 4(*a*), 4(*b*)). Parallel time control cells which had been sham permeabilized exhibited no decrease in whole cell conductance. A similar time course and inhibition was observed for the 20 mer antisense (FIGS. 5(A), 5(B)). Transfection of the sense oligonucleotide produced no significant changes in the whole cell conductance increases associated with hypotonic swelling (FIG. 6). Return of the whole cell conductance response to hypotonicity was observed 26 hr following introduction of the antisense oligonucleotides. This response was significantly higher than the control response, suggesting some feedback mechanism on the gene to increase expression of this protein.

In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

As various changes could be made in the above compositions and methods without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i i i) NUMBER OF SEQUENCES: 17

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7860 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCCAACACCT   TAATATTTAT   ATCTCCACGT   TGTTTTATTT   TTTTTCCCCT   TGTTTTGGGG       60

GGCTTTTATT   TTCCTTTTGT   TTTTAAAATT   TTATCCTTGT   ATATCACAAT   AATGGAAAGA      120

AAGTTTATAG   TGTCTTTCAC   AAAGGAGCGT   AGTTTAAATG   CCCCGCCGCG   CCCGCGCCCG      180

CCCCTGCCAG   AACGGCGCTC   GGCGGCGCGG   CCCGGAGCGG   CGGCGGCGGT   GGCGGCGGCG      240
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GCGGCCGTTC  CCGCGGGCTC  GCCCTCAGGT  GTTCGCGGCT  GCCGTCGCCG  AAGATCGCGG   300

GTCGGGGCCT  CGCGGCGATC  GCCCTGGGCG  GGCCGGAGAC  GCCTCGGGCC  CCCTGGCGGC   360

TCGGGGTCCA  CCCGGCGCCG  CGGGCCCGCC  GCTCTCCCTC  GCCTCGCCTT  TGCGCCTCTT   420

CTCGCTCTGC  CTCTCCATTT  ATTATTATTA  TCATTTTTGT  TTTCAAATGG  TGTAGCCGCC   480

AGAGGTGCGG  TGCTAAATTC  TTGGAAGGGG  CCCGGATGTA  CTGAGGATGC  ATTACAATCT   540

CACGAAAGGA  GGCGGTAGTG  GAAAGCAGCA  GTTTTTGGTG  TTTGGTGCAA  TAATGGGGAT   600

CAGGTAATCA  CCCGAAGGAG  CAAGAACCAC  TGCGGATCCA  CGGCTTCCTG  GATTTGCGCG   660

AGAGCCGCCG  GCCTCGGAGG  AGGGATCCAT  CCAGAGGCTC  GCGGGCTGTT  GCTGCATTTC   720

TTCCTCTTTG  TGGCTTCTCC  TTTCCAAGCA  GTTTTTGGCC  AATGGTCAAT  GAAAACACGA   780

GGATGTACGT  TCCAGAGGAA  AACCACCAAG  GTTCCAACTA  TGGGAGCCCA  CGCCCAGCTC   840

ATGCCAACAT  GAATGCCAAT  GCAGCTGCAG  GACTTGCCCC  CGAGCACATC  CCTACTCCAG   900

GGGCAGCACT  GTCCTGGCAG  GCAGCCATCG  ATGCCGCCCG  GCAGGCCAAG  TTAATGGGCA   960

GTGCTGGCAA  TGCGACCATC  TCTACCGTCA  GTTCCACACA  GCGGAAGCGG  CAGCAGTATG  1020

GGAAACCCAA  GAAGCAGGGG  GGCACAACTG  CCACACGGCC  GCCCCGGGCT  CTGCTCTGCC  1080

TGACTCTGAA  GAACCCCATC  AGGAGGGCAT  GCATCAGCAT  TGTTGAATGG  AAACCATTTG  1140

AAATAATTAT  TTTATTGACG  ATTTTTGCCA  ATTGTGTGGC  CTTAGCAATC  TATATTCCCT  1200

TTCCGGAAGA  TGACTCCAAC  GCCACCAACT  CCAACCTGGA  ACGGGTGGAG  TATCTCTTCC  1260

TCATCATTTT  TACCGTGGAA  GCATTTTTAA  AAGTGATTGC  CTACGGACTA  CTCTTCCACC  1320

CCAACGCTTA  CCTCCGCAAC  GGTTGGAATT  TACTAGATTT  TATAATCGTG  GTTGTAGGGC  1380

TTTTTAGTGC  AATTTTAGAA  CAAGCAACCA  AAGCTGACGG  GGCCAACGCT  CTGGGAGGGA  1440

AAGGAGCGGG  ATTCGATGTG  AAGGCACTGA  GAGCTTTCCG  TGTGCTTCGT  CCCCTGCGGC  1500

TGGTGTCTGG  AGTCCCAAGT  CTCCAGGTGG  TCCTGAACTC  CATCATCAAG  GCCATGGTGC  1560

CCCTGCTGCA  CATTGCCCTC  CTCGTGCTCT  TCGTCATCAT  CATCTATGCC  ATTATCGGCC  1620

TGGAGCTCTT  CATGGGGAAG  ATGCACAAGA  CCTGCTACAA  CCAGGAGGGC  ATAATAGATG  1680

TTCCAGCGGA  AGAGGATCCC  TCCCCTTGTG  CTTTGGAGAC  AGGCCATGGG  CGACAGTGTC  1740

AGAACGGGAC  CGTGTGCAAG  CCCGGGTGGG  ATGGGCCCAA  GCACGGCATC  ACCAACTTCG  1800

ACAACTTCGC  CTTCGCCATG  CTGACGGTGT  TCCAGTGTAT  CACCATGGAG  GGCTGGACAG  1860

ACGTGCTGTA  CTGGATGCAA  GACGCTATGG  GCTATGAGTT  GCCCTGGGTG  TATTTTGTCA  1920

GTCTGGTCAT  CTTTGGATCC  TTTTTCGTTC  TAAATCTGGT  TCTCGGTGTT  TTGAGCGGAG  1980

AGTTTTCCAA  AGAGAGGGAG  AAAGCCAAAG  CTCGAGGAGA  CTTCCAGAAG  CTTCGTGAGA  2040

AGCAGCAGCT  AGAAGAAGAT  CTCAAAGGCT  ACCTGGACTG  GATCACCCAG  GCGGAAGACA  2100

TAGACCCTGA  GAATGAGGAC  GAGGGCATGG  ATGAAGACAA  ACCCCGAAAC  ATGAGCATGC  2160

CCACAAGTGA  GACTGAGTCT  GTCAACACCG  AAAACGTGGC  TGGAGGTGAC  ATCGAGGGTG  2220

AAAACTGTGG  AGCCCGGCTT  GCCCACCGGA  TCTCCAAATC  CAAGTTCAGC  CGCTACTGGC  2280

GCCGGTGGAA  TAGATTCTGC  AGAAGAAAGT  GCCGTGCCGC  AGTTAAGTCC  AACGTCTTCT  2340

ACTGGCTGGT  GATCTTCCTG  GTGTTCCTCA  ACACCCTCAC  CATTGCCTCC  GAACACTACA  2400

ACCAGCCTCA  TTGGCTCACA  GAAGTGCAAG  ACACGGCCAA  CAAGGCCCTC  CTGGCCCTTT  2460

TCACGGCAGA  AATGCTCCTG  AAGATGTACA  GCCTGGGTCT  GCAGGCCTAC  TTTGTATCCC  2520

TCTTCAACCG  CTTTGACTGT  TTCATTGTGT  GCGGGGGCAT  CCTGGAGACC  ATCCTGGTGG  2580

AGACGAAGAT  CATGTCGCCC  CTGGGCATCT  CCGTGCTGAG  ATGTGTGCGG  CTGCTCAGGA  2640
```

```
TTTTCAAGAT  CACCAGGTAC  TGGAACTCCC  TGAGCAACCT  CGTGGCGTCC  TTGCTGAACT    2 7 0 0

CAGTGCGCTC  CATCGCCTCC  CTGCTGCTAC  TCCTCTTCCT  CTTCATCATC  ATCTTCTCCC    2 7 6 0

TCCTGGGGAT  GCAGCTGTTT  GGTGGAAAGT  TCAACTTTGA  TGAGATGCAG  ACCCGTAGGA    2 8 2 0

GCACGTTTGA  TAACTTCCCG  CAGTCTCTCC  TCACTGTGTT  TCAGATCCTG  ACCGGGGAGG    2 8 8 0

ACTGGAATTC  GGTGATGTAT  GATGGGATCA  TGGCTTATGG  CGGCCCCTCT  TTTCCAGGGA    2 9 4 0

TGTTAGTCTG  TATTTACTTC  ATCATCCTCT  TCATCTGTGG  AAATTATATC  CTACTGAATG    3 0 0 0

TGTTCTTGGC  CATTGCGGTG  GACAACCTGG  CTGATGCGGA  GAGCCTGACC  TCTGCCCAAA    3 0 6 0

AAGAGGAAGA  AGAAGAGAAG  GAGAGAAAGA  AGCTGGCCAG  GACTGCCAGC  CCAGAAAAGA    3 1 2 0

AACAGGAGGT  GATGGAGAAA  CCAGCAGTAG  AGGAGAGCAA  AGAGGAGAAA  ATTGAGCTGA    3 1 8 0

AGTCCATTAC  AGCAGATGGA  GAATCCCCGC  CCACTACCAA  GATCAACATG  GATGACCTCC    3 2 4 0

AGCCCAGTGA  AAATGAGGAC  AAGAGTCCCC  ACTCCAACCC  AGACACTGCA  GGTGAAGAGG    3 3 0 0

ATGAGGAGGA  GCCCGAGATG  CCTGTGGGCC  CACGCCCCG  GCCCCTGTCT  GAGCTGCACC    3 3 6 0

TTAAGGAAAA  GGCAGTCCCC  ATGCCGGAAG  CCAGTGCATT  TTTCATCTTC  AGCCCAAACA    3 4 2 0

ACAGGTTCCG  CCTGCAGTGC  CACCGCATTG  TCAATGACAC  GATCTTCACC  AACCTCATCC    3 4 8 0

TCTTCTTCAT  TCTGCTCAGT  AGCATCTCCC  TGGCGGCTGA  GGACCCCGTC  CAGCACACCT    3 5 4 0

CCTTCAGGAA  CCACATCCTA  GGCAATGCAG  ACTATGTCTT  CACTAGTATC  TTTACATTAG    3 6 0 0

AAATTATCCT  TAAGATGACT  GCTTACGGGG  CTTTCCTGCA  CAAGGGCTCT  TTCTGCCGAA    3 6 6 0

ATTACTTCAA  TATCCTGGAC  CTGCTGGTGG  TTAGCGTGTC  CCTCATCTCC  TTTGGCATTC    3 7 2 0

AGTCCAGCGC  GATCAACGTT  GTGAAGATTT  TAAGAGTGCT  TCGTGTCCTC  AGGCCCCTGA    3 7 8 0

GGGCCATCAA  CAGGGCCAAG  GGGCTAAAGC  ACGTCGTTCA  GTGTGTGTTT  GTGGCCATCC    3 8 4 0

GGACCATTGG  AAACATTGTA  ATTGTCACCA  CTCTGCTGCA  GTTCATGTTC  GCCTGCATTG    3 9 0 0

GGGTCCAGCT  CTTCAAGGGA  AAGCTCTATA  CCTGTTCGGA  TAGTTCCAAA  CAGACGGAGG    3 9 6 0

CAGAATGCAA  GGGTAACTAT  ATAACATACA  AAGACGGAGA  AGTTGACCAC  CCCATTATCC    4 0 2 0

AGCCTCGAAG  TTGGGAGAAC  AGCAAGTTCG  ACTTTGACAA  TGTTCTGGCA  GCCATGATGG    4 0 8 0

CCCTCTTTAC  CGTCTCCACC  TTCGAGGGGT  GGCCAGAGCT  GCTGTACCGC  TCCATTGACT    4 1 4 0

CCCACACAGA  AGACAAGGGT  CCCATCTACA  ACTATCGTGT  GGAGATCTCC  ATCTTCTTCA    4 2 0 0

TCATCTACAT  CATCATCATT  GCCTTCTTCA  TGATGAACAT  CTTCGTGGGT  TTCGTCATTG    4 2 6 0

TCACCTTCCA  GGAGCAGGGG  GAACAAGAAT  ACAAGAACTG  TGAGCTGGAC  AAGAACCAGA    4 3 2 0

GACAATGTGT  GGAATATGCC  CTCAAGGCCC  GACCCTTGCG  AAGGTACATC  CCCAAGAACC    4 3 8 0

AGCACCAGTA  CAAAGTGTGG  TACGTGGTCA  ACTCCACCTA  CTTCGAGTAT  CTGATGTTCG    4 4 4 0

TCCTCATCCT  GCTCAACACC  ATCTGCCTGG  CCATGCAGCA  CTACGGCCAG  AGCTGCCTCT    4 5 0 0

TCAAAATCGC  CATGAATATA  CTCAACATGC  TTTTCACTGG  CCTCTTCACG  GTGGAGATGA    4 5 6 0

TCCTGAAGCT  CATTGCCTTC  AAACCCAAGG  GTTACTTTAG  TGATCCCTGG  AATGTTTTTG    4 6 2 0

ACTTCCTCAT  CGTCATTGGG  AGCATAATTG  ATGTCATTCT  CAGTGAAACT  AATCCAGCTG    4 6 8 0

AACATACCCA  ATGCTCTCCC  TCTATGAGTG  CAGAGGAGAA  CTCCCGCATC  TCCATCACCT    4 7 4 0

TCTTCCGCCT  CTTCCGGGTC  ATGCGCCTGG  TGAAGCTGCT  GAGCCGAGGG  GAAGGCATCC    4 8 0 0

GGACCCTGCT  GTGGACCTTC  ATCAAGTCCT  TCCAGGCCCT  GCCCTATGTG  GCCCTTTTGA    4 8 6 0

TTGTGATGCT  GTTCTTTATC  TATGCAGTGA  TTGGGATGCA  GGTATTTGGG  AAGATTGCCC    4 9 2 0

TGAATGACAC  CACAGAGATC  AATCGGAACA  ACAACTTCCA  GACGTTCCCT  CAGGCTGTGC    4 9 8 0

TACTGCTCTT  CAGGTGCGCC  ACTGGGGAGG  CCTGGCAGGA  TATCATGCTA  GCCTGTATGC    5 0 4 0
```

```
CAGGCAAGAA  GTGTGCTCCA  GAGTCTGAGC  CCAGCAACAG  CACGGAAGGG  GAGACACCCT  5100
GTGGCAGCAG  TTTCGCTGTC  TTCTACTTCA  TCAGCTTCTA  CATGCTCTGT  GCCTTCCTGA  5160
TCATCAACCT  CTTTGTAGCT  GTTATCATGG  ACAACTTTGA  CTACCTGACT  AGGGATTGGT  5220
CTATCCTTGG  TCCCCATCAC  CTGGATGAAT  TCAAGAGAAT  CTGGGCCGAA  TATGACCCTG  5280
AAGCCAAGGG  TCGGATCAAA  CACTTGGATG  TGGTGACCCT  CCTCCGTCGA  ATTCAGCCCC  5340
CACTGGGTTT  TGGGAAGTTG  TGTCCTCACC  GTGTGGCCTG  CAAACGCCTG  GTGTCCATGA  5400
ACATGCCTCT  GAACAGTGAT  GGGACGGTCA  TGTTCAATGC  TACACTGTTT  GCCCTTGTCA  5460
GGACAGCCCT  GAGGATCAAA  ACAGAAGGGA  ACCTAGAGCA  AGCCAATGAG  GAGCTGAGAG  5520
CCATCATCAA  GAAAATCTGG  AAGAGGACCA  GCATGAAGCT  GTTGGACCAG  GTGGTGCCCC  5580
CTGCAGGTGA  TGACGAGGTC  ACAGTGGGCA  AGTTCTATGC  CACCTTCCTG  ATCCAAGAGT  5640
ACTTCAGGAA  ATTCAAGAAG  CGAAAAGAGC  AGGGGCTGGT  CGGCAAGCCC  TCGCAGAGGA  5700
ATGCACTGTC  TCTGCAGGCT  GGCTTACGCA  CCTTGCATGA  CATTGGGCCT  GAGATCCGGA  5760
GAGCCATCTC  TGGGGATCTG  ACGGCTGAGG  AGGAGCTGGA  CAAGGCTATG  AAGGAGGCAG  5820
TGTCTGCTGC  CTCCGAAGAC  GACATCTTCA  GGAGGGCTGG  AGGCCTGTTT  GGCAACCACG  5880
TCAGCTACTA  CCAGAGTGAC  AGCAGGAGCA  ACTTCCCTCA  GACGTTTGCC  ACCCAACGCC  5940
CACTGCACAT  CAACAAGACA  GGGAACAACC  AAGCGGACAC  CGAATCACCG  TCCCATGAGA  6000
AGCTGGTGGA  CTCCACTTTC  ACCCCCAGCA  GCTACTCATC  CACGGGCTCC  AATGCCAACA  6060
TCAACAATGC  CAACAACACT  GCCCTGGGCC  GCTTCCCCCA  CCCTGCTGGC  TACTCCAGCA  6120
CGGTCAGCAC  TGTGGAGGGC  CATGGGCCTC  CCTTGTCCCC  TGCTGTCCGG  GTACAGGAGG  6180
CAGCATGGAA  ACTCAGCTCT  AAGAGGTGCC  ACTCCCGAGA  GAGCCAGGGG  GCCACGGTGA  6240
GTCAGGATAT  GTTTCCAGAT  GAGACCCGCA  GCAGCGTGAG  GCTGAGCGAA  GAAGTTGAGT  6300
ACTGCAGTGA  GCCCAGCCTG  CTCTCCACAG  ATATACTCTC  CTACCAGGAC  GATGAAAACC  6360
GACAACTGAC  CTGTCTAGAG  GAGGACAAGA  GGGAGATCCA  GCCATCTCCG  AAGAGGAGTT  6420
TCCTTCGCTC  TGCCTCTCTA  GGTCGAAGGG  CCTCCTTCCA  TCTGGAATGT  CTAAAGCGAC  6480
AAAAGGATCA  AGGAGGAGAC  ATCTCTCAGA  AGACAGCCTT  GCCCTTGCAT  CTGGTTCATC  6540
ACCAGGCATT  GGCAGTGGCA  GGCTTGAGCC  CCCTCCTGCA  GAGAAGCCAT  TCTCCTTCCA  6600
CGTTTCCCAG  GCCACGCCCC  ACGCCCCCTG  TCACTCCAGG  CAGCCGGGGC  AGGCCCCTAC  6660
AGCCCATCCC  TACCCTGCGG  CTGGAGGGGG  CGGAGTCCAG  TGAGAAACTC  AACAGCAGCT  6720
TCCCGTCCAT  CCACTGCAGC  TCCTGGTCAG  AGGAGACCAC  AGCCTGTAGT  GGGGGCAGCA  6780
GCATGGCCCG  GAGAGCCCGG  CCCGTCTCCC  TCACCGTGCC  CAGCCAGGCT  GGAGCTCCAG  6840
GCAGACAGTT  CCACGGCAGC  GCCAGCAGCC  TGGTGGAAGC  GGTCTTGATT  TCAGAAGGAC  6900
TGGGACAGTT  TGCTCAAGAT  CCCAAGTTCA  TCGAGGTCAC  CACACAGGAG  CTGGCTGACG  6960
CCTGCGATAT  GACAATAGAG  GAGATGGAGA  ACGCCGCAGA  CAACATCCTC  AGCGGGGGCG  7020
CCCAGCAGAG  CCCCAACGGC  ACCCTCTTAC  CTTTTGTGAA  CTGCAGGGAC  CCAGGGCAGG  7080
ACAGGGCTGT  GGTCCCAGAG  GACGAGAGCT  GTGTATATGC  CCTGGGGCGA  GGCCAGAGCG  7140
AGGAAGCGCT  CCCCGGACAGC  AGGTCCTATG  TCAGCAACCT  GTAGTCCGCA  GGGCTGGCGA  7200
GACGCGGGTG  TTTTTTATTC  GTTTCAATGT  TCCTAATGGG  TTCGTTTCAG  AAGTGCCTCA  7260
CTGTTCTCGT  GACCTGGAGG  TAACCGGAAC  AGCGTCTTCA  TTCACTGCTG  TCGGGATAAG  7320
CCTCAGAGCT  GGGCGGTGTA  CGAAGTCGGC  TTTTCAGGGG  AGAAGGCCAA  GGCCGTGGTG  7380
CGGGGGCTCC  AGCACCTTCC  CACGGCAGCA  CCGCCCAAAG  GACCCCACCC  CCCCTAAGCA  7440
```

```
AAAGGGTGTT  TTCCCCTTGC  TTGTATAAAC  AGTCATTTGC  ACATGTTCTG  TCTGAGCCTG      7500

GCCGTCTCTA  TGGAGCAGGG  CCCCAGGGAT  CTATGGCAGG  AATGGCCAGT  GTCCCCAGTA      7560

GGAGCCGGAA  GGTGGCTGCA  AGGTTCCCAG  CAGTGCAGAT  CTGGTCCCTA  TTGCCCTTCA      7620

GGGACCCTTC  CCCTGCTGGA  ACTGAGGAGC  AGGTGCAGGA  GCCAGTGCAG  ACCACACCAC      7680

CTGCCCTCAG  CTAGCCAGAC  CTGGGGGCGC  AGGCTGCTTG  CCTGGTGCTC  TGGGTTTCAT      7740

AGTTTGATGG  TTCTTGTCAG  CATGTTGCGG  TTTTCTAGGT  TTTGATTTCT  TTATTACTAT      7800

TTGTTGTGTT  TTCCCACGGG  GAGGGGAGGA  AGAAGAGCGT  TTACAACTGC  GCAGCTCACG      7860
```

(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CCTTCCGTGC  TGTTGCTGGG  CTCA                                                  24
```

(2) INFORMATION FOR SEQ ID NO:3:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ACTCTGGAGC  ACACTTCTTG                                                        20
```

(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 844 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GGGGGAGACT  GAGTATAAGA  ACTGTGAGCT  GGACAAGAAC  CAGCGCCAAT  GTGTGCAGTA        60

TGCCCTGAAG  GCCCGCCCGC  TGAGGTGTTA  CATCCCCAAA  AACCCATACC  AGTATCAGGT       120

GTGGTATGTC  GTCACTTCCT  CCTACTTTGA  ATACCTGATG  TTTGCTCTCA  TCATGCTCAA       180

CACTATCTGC  CTAGGCATGC  AGCATTACAA  CCAGTCGGAA  CAGATGAACC  ACATCTCGGA       240

CATCCTCAAT  GTGGCTTTCA  CCATCATCTT  CACCCTGGAG  ATGATCCTCA  AGCTCATAGC       300

TTTCAAGCCC  AGGGGCTATT  TTGGAGACCC  CTGGAACGTG  TTTGACTTTC  TAATCGTCAT       360

TGGCAGCATC  ATTGACGTTA  TCCTGAGTGA  GATCGATGAC  CCAGATGAGA  GCGCCGCAT       420

CTCCAGTGCC  TTCTTCCGCC  TGTTCCGAGT  CATGCGACTG  ATCAAGCTGC  TGAGCCGGGC       480

GGAGGGTGTG  CGCACCCTGC  TCTGGACGTT  CACCAAGTCC  TTCCAGGCCC  TGCCGTATGT       540

GGCTTTGCTG  ATCGTCATGC  TCTTCTTCAT  CTACGCTGTC  ATCGGCATGC  AGATGTTCGG       600

GAAGATCGCC  ATGGTGGACG  GGACGCAAAT  AAACCGGAAC  AACAACTTCC  AGACTTTCCC       660

ACAAGCTGTG  CTGCTGCTCT  TCAGGTGTGC  CACAGGGGCA  GCCTGGCAGG  AGATCCTGCT       720

GGCCTGCAGC  TACGGGAAAC  GCTGCGACCC  GGAGTCTGAC  TACGCACCGG  GGGAGGAGTA       780

CGCGTGTGGC  ACCAACTTTG  CCTACTACTA  CTTCATCAGC  TTCTACATGC  TCTGCGCCTT       840
```

C C T G 844

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 850 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GGGGGAACAA  GAATACAAGA  ACTGTGAGCT  GGACAAGAAC  CAGAGACAAT  GTGTGGAATA      60
TGCCCTCAAG  GCCCGACCCT  TGCGAAGGTA  CATCCCCAAG  AACCAGCACC  AGTACAAAGT     120
GTGGTACGTG  GTCAACTCCA  CCTACTTCGA  GTATCTGATG  TTCGTCCTCA  TCCTGCTCAA     180
CACCATCTGC  CTGGCCATGC  AGCACTACGG  CCAGAGCTGC  CTCTTCAAAA  TCGCCATGAA     240
TATACTCAAC  ATGCTTTTCA  CTGGCCTCTT  CACGGTGGAG  ATGATCCTGA  AGCTCATTGC     300
CTTCAAACCC  AAGGGTTACT  TTAGTGATCC  CTGGAATGTT  TTTGACTTCC  TCATCGTCAT     360
TGGGAGCATA  ATTGATGTCA  TTCTCAGTGA  AACTAATAGT  GCAGAGGAGA  ACTCCCGCAT     420
CTCCATCACC  TTCTTCCGCC  TCTTCCGGGT  CATGCGCCTG  GTGAAGCTGC  TGAGCCGAGG     480
GGAAGGCATC  CGGACCCTGC  TGTGGACCTT  CATCAAGTCC  TTCCAGGCCC  TGCCCTATGT     540
GGCCCTTTTG  ATTGTGATGC  TGTTCTTTAT  CTATGCAGTG  ATTGGGATGC  AGGTATTTGG     600
GAAGATTGCC  CTGAATGACA  CCACAGAGAT  CAATCGGAAC  AACAACTTCC  AGACGTTCCC     660
TCAGGCTGTG  CTACTGCTCT  TCAGGTGCGC  CACTGGGGAG  GCCTGGCAGG  ATATCATGCT     720
AGCCTGTATG  CCAGGCAAGA  AGTGTGCTCC  AGAGTCTGAG  CCCAGCAACA  GCACGGAAGG     780
GGAGACACCC  TGTGGCAGCA  GTTTCGCTGT  CTTCTACTTC  ATCAGCTTCT  ACATGCTCTG     840
TGCCTTCCTG                                                              850
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 844 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GGGAGAAAAG  GAGTATAAGA  ACTGTGAGCT  GGACAAAAAT  CAGCGTCAGT  GTGTGGAATA      60
TGCCTTGAAG  GCCCGCCCCT  TAAGGAGATA  CATCCCCAAA  AACCCATACC  AGTACAAGTT     120
CTGGTACGTG  GTGAACTCCT  CGCCTTTCGA  ATATATGATG  TTTGTCCTCA  TCATGCTCAA     180
CACGCTCTGC  CTGGCCATGC  AGCACTATGA  GCAATCCAAG  ATGTTCAATG  ACGCCATGGA     240
CATTCTGAAC  ATGGTCTTCA  CGGGGGTCTT  CACCGTTGAG  ATGGTTTTGA  AAGTCATCGC     300
ATTTAAGCCC  AAGGGGTATT  TTAGTGACGC  CTGGAACACG  TTTGACTCCC  TCATCGTAAT     360
CGGCAGCATT  ATAGACGTGG  CACTCAGCGA  AGCTGACAAC  TCTGAAGAGA  GCAATAGAAT     420
CTCCATCACC  TTTTTCCGTC  TTTTCCGAGT  GATGCGGTTG  GTGAAGCTTC  TCAGCAGAGG     480
GGAAGGCATC  CGGACTCTGC  TATGGACCTT  CATTAAGTCC  TTCCAGGCAC  TCCCATATGT     540
CGCCCTCCTC  ATTGCCATGC  TCTTCTTCAT  CTACGCCGTC  ATTGGCATGC  AGATGTTTGG     600
GAAGGTTGCC  ATGAGAGATA  ACAACCAGAT  CAATAGGAAC  AACAACTTCC  AGACGTTTCC     660
CCAGGCAGTG  CTGCTGCTCT  TCAGGTGTGC  AACAGGGGAG  GCCCGGCAGG  AGATCATGCT     720
CGCCTGCCTC  CCTGGGAAGC  TGTGTGACCC  GGACTCAGAT  TACAACCCAG  GAGAGGAATA     780
```

TACTTGTGGG AGCAACTTTG CCATTGTCTA CTTCATCAGC TTTTACATGC TCTGCGCGTT   840

CCTG   844

(2) INFORMATION FOR SEQ ID NO:7:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 51 amino acid residues
                    (B) TYPE: amino acid
                    (D) TOPOLOGY: linear

        (x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

          Pro   Trp   Asn   Val   Phe   Asp   Phe   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
          1   5   10   15

          Val   Ile   Leu   Ser   Glu   Ile   Asp   Asp   Pro   Asp   Glu   Ser   Ala   Arg   Ile   Ser
                            20   25   30

          Ser   Ala   Phe   Phe   Arg   Leu   Phe   Arg   Val   Met   Arg   Leu   Ile   Lys   Leu   Leu
                            35   40   45

          Ser   Arg   Ala
                      50

(2) INFORMATION FOR SEQ ID NO:8:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 70 amino acid residues
                    (B) TYPE: amino acid
                    (D) TOPOLOGY: linear

        (x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

          Pro   Trp   Asn   Val   Phe   Asp   Phe   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
          1   5   10   15

          Val   Ile   Leu   Ser   Glu   Ile   Asp   Thr   Phe   Leu   Ala   Ser   Ser   Gly   Gly   Leu
                            20   25   30

          Tyr   Cys   Leu   Gly   Gly   Gly   Cys   Gly   Asn   Val   Asp   Pro   Asp   Glu   Ser   Ala
                            35   40   45

          Arg   Ile   Ser   Ser   Ala   Phe   Phe   Arg   Leu   Phe   Arg   Val   Met   Arg   Leu   Ile
                      50   55   60

          Lys   Leu   Leu   Ser   Arg   Ala
          65   70

(2) INFORMATION FOR SEQ ID NO:9:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 51 amino acid residues
                    (B) TYPE: amino acid
                    (D) TOPOLOGY: linear

        (x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

          Pro   Trp   Asn   Val   Phe   Asp   Phe   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
          1   5   10   15

          Val   Ile   Leu   Ser   Glu   Thr   Asn   Ser   Ala   Glu   Glu   Asn   Ser   Arg   Ile   Ser
                            20   25   30

          Ile   Thr   Phe   Phe   Arg   Leu   Phe   Arg   Val   Met   Arg   Leu   Val   Lys   Leu   Leu
                            35   40   45

          Ser   Arg   Gly
                      50

(2) INFORMATION FOR SEQ ID NO:10:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 62 amino acid residues
                    (B) TYPE: amino acid
                    (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Pro   Trp   Asn   Val   Phe   Asp   Phe   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
1                       5                             1 0                            1 5

Val   Ile   Leu   Ser   Glu   Thr   Asn   Pro   Ala   Glu   His   Thr   Gln   Cys   Ser   Pro
                  2 0                      2 5                            3 0

Ser   Met   Ser   Ala   Glu   Glu   Asn   Ser   Arg   Ile   Ser   Ile   Thr   Phe   Phe   Arg
            3 5                      4 0                      4 5

Leu   Phe   Arg   Val   Met   Arg   Leu   Val   Lys   Leu   Leu   Ser   Arg   Gly
      5 0                      5 5                      6 0
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 51 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Ala   Trp   Asn   Thr   Phe   Asp   Ser   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
1                       5                             1 0                            1 5

Val   Ala   Leu   Ser   Glu   Ala   Asp   Asn   Ser   Glu   Glu   Ser   Asn   Arg   Ile   Ser
                  2 0                      2 5                            3 0

Ile   Thr   Phe   Phe   Arg   Leu   Phe   Arg   Val   Met   Arg   Leu   Val   Lys   Leu   Leu
            3 5                      4 0                      4 5

Ser   Arg   Gly
      5 0
```

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 66 amino acid residues
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Ala   Trp   Asn   Thr   Phe   Asp   Ser   Leu   Ile   Val   Ile   Gly   Ser   Ile   Ile   Asp
1                       5                             1 0                            1 5

Val   Ala   Leu   Ser   Glu   Ala   Asp   Pro   Ser   Asp   Ser   Glu   Asn   Ile   Pro   Leu
                  2 0                      2 5                            3 0

Pro   Thr   Ala   Thr   Pro   Gly   Asn   Ser   Glu   Glu   Ser   Asn   Arg   Ile   Ser   Ile
            3 5                      4 0                      4 5

Thr   Phe   Phe   Arg   Leu   Phe   Arg   Val   Met   Arg   Leu   Val   Lys   Leu   Leu   Ser
      5 0                      5 5                      6 0

Arg   Gly
6 5
```

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCCGGATCCA   TCGTCACCTT   CCAGGAGCA   2 9

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs

-continued

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

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATGGAATTCG CCACRAAGAG GTTGATGAT   2 9

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTGGGAATTC ATCAAGTCCT TCCAGGCCCT   3 0

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGGGGATCC AAGTTGTCCA TGATAACAGC   3 0

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCCGAATTCA KMGTGTTGAG CATGATGAG   2 9

What is claimed is:

1. An antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits strain-induced increase in whole cell conductance in osteoblasts, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

2. The antisense oligonucleotide of claim 1, which is DNA.

3. The antisense oligonucleotide of claim 1 in which at least one of the internucleotide linkages is a phosphorothioate phosphodiester.

4. The antisense oligonucleotide of claim 1 further characterized in that it does not hybridize with a CaCh1 or CaCh3 mRNA.

5. A pharmaceutical composition comprising at least one antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits strain-induced increase in whole cell conductance in osteoblasts, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3, and a pharmaceutically acceptable carrier.

6. The pharmaceutical composition of claim 5, wherein the antisense oligonucleotide is DNA.

7. The pharmaceutical composition of claim 5, wherein at least one of the internucleotide linkages is a phosphorothioate phosphodiester.

8. A method of inhibiting expression of a voltage-gated calcium channel α_1 subunit gene in a cell, the method comprising the steps of:

(a) obtaining an antisense oligonucleotide that hybridizes with a region of a voltage-gated calcium channel α_1 subunit mRNA and inhibits expression of said α_1 subunit, having a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3,

(b) combining said antisense oligonucleotide with a pharmaceutically acceptable carrier to create a pharmaceutically active mixture, and

(c) contacting said voltage-gated calcium channel α_1 subunit mRNA within said cell in vitro with said pharmaceutically active mixture, thereby inhibiting expression of said voltage-gated calcium channel α_1 subunit gene within said cell.

9. The method of claim 8, wherein said cell is an osteoblast or an endothelial cell.

10. A method of inhibiting expression of a voltage-gated calcium channel α_1 subunit in a cell, comprising contacting said cell in vitro with an oligonucleotide having the sequence shown in SEQ ID NO:2 or SEQ ID NO:3.

11. The method of claim 10, wherein said cell is an osteoblast or an endothelial cell.

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