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(54) Title: MODULATION OF NOTCH3 EXPRESSION

(57) Abstract: Compounds, compositions and methods are provided for modulating the expression of Notch3. The compositions comprise oligonucleotides, targeted to nucleic acid encoding Notch3. Methods of using these compounds for modulation of Notch3 expression and for diagnosis and treatment of disease associated with expression of Notch3 are provided.

MODULATION OF NOTCH3 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of Notch3. In particular, this invention relates to compounds, particularly oligomeric compounds such as oligonucleotide compounds, which, in some embodiments, hybridize with nucleic acid molecules encoding Notch3. Such compounds are shown herein to modulate the expression of Notch3.

BACKGROUND OF THE INVENTION

Intrinsic, cell-autonomous factors as well as non-autonomous, short-range and long-range signals guide cells through distinct developmental paths. An organism frequently uses the same signaling pathway within different cellular contexts to achieve unique developmental goals.

Notch signaling is an evolutionarily conserved mechanism used to control cell fates through local cell interactions. The gene encoding the original Notch receptor was discovered in *Drosophila melanogaster* due to the fact that partial loss of function of the gene results in notches at the wing margin (Artavanis-Tsakonas et al., Science, 1999, 284, 770-776). Signals transmitted through the Notch receptor, in combination with other cellular factors, influence differentiation, proliferation and apoptotic events at all stages of development (Artavanis-Tsakonas et al., Science, 1999, 284, 770-776).

Mature Notch proteins are heterodimeric receptors derived from the cleavage of Notch pre-proteins into an extracellular subunit containing multiple EGF-like repeats and a transmembrane subunit including the intracellular region (Blaumueller et al., Cell, 1997, 90, 281-291). Notch activation results from the binding of ligands expressed by neighboring cells or soluble ligands and signaling from activated Notch involves networks of transcription regulators (Artavanis-Tsakonas et al., Science, 1995, 268, 225-232).

In context of experimental cancer immunotherapy, the Notch signaling network is acquiring increasing importance for its possible roles in neoplastic cells and the immune system (Jang et al., Curr. Opin. Mol. Ther., 2000, 2, 55-65). Larsson et al. predicted that the human Notch genes are proto-oncogenes and candidates for sites of chromosome breakage in neoplasia-associated translocations (Larsson et al., Genomics, 1994, 24, 253-258).

Four mammalian Notch homologs have been identified and are designated Notch1, Notch2, Notch3 and Notch4. Human Notch3 (also known as Notch (Drosophila) homolog 3 and CADASIL) was identified and mapped to chromosome 19p13.2-p13.1, a region associated with neoplasia-associated translocations (Larsson et al., Genomics, 1994, 24, 253-258).

Disclosed and claimed in US Patent 5,789,195 are nucleic acid sequences encoding Notch genes. Antibodies to human Notch proteins are additionally provided (Artavanis-Tsakonas et al., 1998). Amino acid sequences of Notch genes and antibodies against Notch proteins are also disclosed and claimed in US Patent 6,090,922 (Artavanis-Tsakonas et al., 2000).

Mutations in Notch3 have been identified as the cause of CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), an autosomal dominant condition whose key features include recurrent subcortical ischemic strokes which lead to progressive dementia (Joutel and Tournier-Lasserve, Semin. Cell Dev. Biol., 1998, 9, 619-625).

Modulation of expression of Notch genes may prove to be a useful point for therapeutic intervention in developmental, hyperproliferative or autoimmune disorders or disorders arising from aberrant apoptosis.

Methods for genotypic diagnosis of CADASIL wherein the DNA of a symptomatic or at risk individual is contacted with primer pairs which specifically hybridize to Notch3 (Joutel et al., 1997).

Therapeutic methods utilizing anti-Notch3 antibodies and antisense sequences which block expression of Notch3 are disclosed and claimed in PCT publication WO 98/05775 (Tournier-Lasserve et al., 1998).

Methods for producing allergen- or antigen-tolerant T-cells employing compositions capable of upregulating expression of an endogenous Notch protein are disclosed and claimed in PCT publication WO 00/36089 (Lamb et al., 2000).

Disclosed and claimed in US Patent 6,149,902 is a method for cell transplantation which includes contacting a precursor cell with an agonist of Notch function effective to inhibit differentiation of the cell wherein said agonist is a Delta protein, a Serrate protein or an antibody to a Notch protein (Artavanis-Tsakonas et al., 2000).

Disclosed in US Patent 6,083,904 and PCT publication WO 94/07474 are therapeutic and diagnostic methods and compositions based on Notch proteins and nucleic acids, wherein antisense methods are generally disclosed (Artavanis-Tsakonas, 2000; Artavanis-Tsakonas et al., 1994).

Disclosed and claimed in US Patent 5,786,158 are methods and compositions for the detection of malignancy or nervous system disorders based on the level of Notch proteins or nucleic acids (Artavanis-Tsakonas et al., 1998).

Disclosed and claimed in PCT publication WO 00/20576 are methods for inducing differentiation and apoptosis in human cells that over express Notch proteins wherein Notch function is disrupted using antisense oligonucleotides that target the EGF repeat region, the lin/notch region and the ankyrin region (Miele et al., 2000).

Disclosed and claimed in PCT publication WO 01/25422 is an antisense oligonucleotide directed to exon 30 of human Notch3 (Bartelmez and Iversen, 2001).

Currently, there are no known therapeutic agents that effectively inhibit the synthesis of Notch3. To date, investigative strategies aimed at modulating Notch3 expression have involved the use of antibodies and Notch-regulating proteins as well as antisense RNA and oligonucleotides. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting Notch3 function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of expression of Notch3.

The present invention provides compositions and methods for modulating expression of Notch3.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, especially nucleic acid and nucleic acid-like oligomers, which are targeted to a nucleic acid encoding Notch3, and which modulate the expression of Notch3. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of screening for modulators of Notch3 and methods of modulating the expression of Notch3 in cells, tissues or animals comprising contacting said cells, tissues or animals with one or more of the compounds or compositions of the invention. Methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of Notch3 are also set forth herein. Such methods comprise administering a therapeutically or prophylactically effective amount of one or more of the compounds or compositions of the invention to the person in need of treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs compounds, including oligomers such as oligonucleotides and similar species for use in modulating the function or effect of nucleic acid molecules encoding Notch3. This is accomplished by providing oligonucleotides that specifically hybridize with one or more nucleic acid molecules encoding Notch3. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding Notch3" have been used for convenience to encompass DNA encoding Notch3, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The hybridization of a compound of this invention with its target nucleic acid is generally referred to as "antisense." Consequently, a mechanism believed to be included in the practice of some embodiments of the invention is referred to herein as "antisense inhibition." Such antisense inhibition is typically based upon hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard, specific nucleic acid molecules and their functions can be targeted for such antisense inhibition.

The functions of DNA to be interfered with include, but are not limied to, replication and transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. Functions of RNA to be interfered with also include functions such as, for example, translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA. One result of such interference with target nucleic acid function is modulation of the expression of Notch3. In the context of the present invention, "modulation" and "modulation of expression" mean either an increase (stimulation) or a decrease (inhibition) in the amount or levels of a nucleic acid molecule encoding the gene, e.g., DNA or RNA. Inhibition is often a desired form of modulation of expression and mRNA is often a desired target nucleic acid.

In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, one mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases that

pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

The compounds of the invention are specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity. In some embodiments, there may be a sufficient degree of complementarity to avoid non-specific binding of the compound to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances and in the context of this invention, "stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleobases of an oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, the target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

It is understood in the art that the sequence of a compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). The compounds of the present invention can comprise at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence complementarity to a target

region within the target nucleic acid sequence to which they are targeted. For example, a compound in which 18 of 20 nucleobases of the compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, a compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would fall within the scope of the present invention. Percent complementarity of a compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; and Zhang and Madden, Genome Res., 1997, 7, 649-656).

Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489). In some embodiments, homology, sequence identity or complementarity, between the oligomeric compound and target is between about 50% to about 60%, between about 60% to about 70%, between about 70% and about 80%, or between about 80% and about 90%. In other embodiments, homology, sequence identity or complementarity, is about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%.

According to the present invention, compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds that hybridize to at least a portion of the target nucleic acid. As such, these compounds may be introduced in the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid.

One non-limiting example of such an enzyme is RNAse H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNAse H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of

oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

While one form of an antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing.

The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, Cell, 1995, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA, 1998, 95, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., Nature, 1998, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., Science, 2002, 295, 694-697).

The oligonucleotides of the present invention also include variants in which a different base is present at one or more of the nucleotide positions in the oligonucleotide. For example, if the first nucleotide is an adenosine, variants may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of Notch3 mRNA.

In the context of this invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions that function similarly. Such modified or substituted oligonucleotides are often favorable over native forms because of desirable properties such as, for example, enhanced

cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

While oligonucleotides are one form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those described herein.

The compounds in accordance with this invention can comprise from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 linked nucleosides). One of ordinary skill in the art will appreciate that the invention embodies compounds of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleobases in length.

In one embodiment, the compounds of the invention are 12 to 50 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleobases in length.

In another embodiment, the compounds of the invention are 15 to 30 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length.

In other embodiments, the compounds are oligonucleotides from about 12 to about 50 nucleobases or from about 15 to about 30 nucleobases.

Antisense compounds 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative compounds are considered to be suitable compounds as well.

Exemplary compounds include oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately upstream of the 5'-terminus of the compound that is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). Similarly, compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately downstream of the 3'-terminus of the compound that is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80

nucleobases). One having skill in the art armed with the compounds illustrated herein will be able, without undue experimentation, to identify additional compounds.

"Targeting" a compound to a particular nucleic acid molecule, in the context of this invention, can be a multistep process. The process can begin with the identification of a target nucleic acid whose function is to be modulated. This target nucleic acid may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target nucleic acid molecule encodes Notch3.

The targeting process can also include determination of at least one target region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect, e.g., modulation of expression, will result. Within the context of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid.

Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding Notch3, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous

nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination codon region") are all regions which may be targeted effectively with the compounds of the present invention.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Within the context of the present invention, a suitable region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. The 5' cap region can be targeted.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also suitable target sites. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts." It is also known that introns can be effectively targeted using antisense compounds targeted to, for example, DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants."

More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequence.

Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants." Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice variants." If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also suitable target nucleic acids.

Locations on the target nucleic acid to which the compounds hybridize are hereinbelow referred to as "suitable target segments." As used herein, the term "suitable target segment" is defined as at least an 8-nucleobase portion of a target region to which an active compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent portions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of particular suitable target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional suitable target segments may be identified by one having ordinary skill.

Once one or more suitable target regions, segments or sites have been identified, compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

The oligomeric compounds are also targeted to or not targeted to regions of the target nucleobase sequence (e.g., such as those disclosed in Example 13) comprising nucleobases 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550,

551-600, 601-650, 651-700, 701-750, 751-800, 801-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, 1251-1300, 1301-1350, 1351-1400, 1401-1450, 1451-1500, 1501-1550, 1551-1600, 1601-1650, 1651-1700, 1701-1750, 1751-1800, 1801-1850, 1851-1900, 1901-1950, 1951-2000, 2001-2050, 2051-2100, 2101-2150, 2151-2200, 2201-2250, 2251-2300, 2301-2350, 2351-2400, 2401-2450, 2451-2500, 2501-2550, 2551-2600, 2601-2650, 2651-2700, 2701-2750, 2751-2800, 2801-2850, 2851-2900, 2901-2950, 2951-3000, 3001-3050, 3051-3100, 3101-3150, 3151-3200, 3201-3250, 3251-3300, 3301-3350, 3351-3400, 3401-3450, 3451-3500, 3501-3550, 3551-3600, 3601-3650, 3751-3700, 3701-3750, 3751-3800, 3801-3850, 3851-3900, 3901-3950, 3951-4000, 4001-4050, 4051-4100, 4101-4150, 4151-4200, 4201-4250, 4251-4300, 4301-4350, 4351-4400, 4401-4450, 4451-4500, 4501-4550, 4551-4600, 4601-4650, 4751-4700, 4701-4750, 4751-4800, 4801-4850, 4851-4900, 4901-4950, 4951-5000, 5001-5050, 5051-5100, 5101-5150, 5151-5200, 5201-5250, 5251-5300, 5301-5350, 5351-5400, 5401-5450, 5451-5500, 5501-5550, 5551-5600, 5601-5650, 5751-5700, 5701-5750, 5751-5800, 5801-5850, 5851-5900, 5901-5950, 5951-6000, 6001-6050, 6051-6100, 6101-6150, 6151-6200, 6201-6250, 6251-6300, 6301-6350, 6351-6400, 6401-6450, 6451-6500, 6501-6550, 6551-6600, 6601-6650, 6651-6700, 6701-6750, 6751-6800, 6801-6850, 6851-6900, 6901-6950, 6951-7000, 7001-7050, 7051-7100, 7101-7150, 7151-7200, 7201-7250, 7251-7300, 7301-7350, 7351-7400, 7401-7450, 7451-7500, 7501-7550, 7551-7600, 7601-7650, 7651-7700, 7701-7750, 7751-7800, 7801-7850, 7851-7900, 7901-7950, 7951-8000, 8001-8050, 8051-8100, 8101-8150, 8151-8200, 8201-8250, 8251-8300, 8301-8350, 8351-8400, 8401-8450, 8451-8500, 8501-8550, 8551-8600, 8601-8650, 8651-8700, 8701-8750, 8751-8800, 8801-8850, 8851-8900, 8901-8950, 8951-9000, 9001-9050, 9051-9100, 9101-9150, 9151-9200, 9201-9250, 9251-9300, 9301-9350, 9351-9400, 9401-9450, 9451-9500, 9501-9550, 9551-9600, 9601-9650, 9651-9700, 9701-9750, 9751-9800, 9801-9850, 9851-9900, 9901-9950, 9951-10000, 10001-10050, 10051-10100, 10101-10150, 10151-10200, 10201-10250, 10251-10300, 10301-10350, 10351-10400, 10401-10450, 10451-10500, 10501-10550, 10551-10600, 10601-10650, 10651-10700, 10701-10750, 10751-10800, 10801-10850, 10851-10900, 10901-10950, 10951-11000, 11001-11050, 11051-11100, 11101-11150, 11151-11200, 11201-11250, 11251-11300, 11301-11350, 11351-11400, 11401-11450, 11451-11500, 11501-11550, 11551-11600, 11601-11650, 11651-11700, 11701-11750, 11751-11800, 11801-11850, 11851-11900, 11901-11950, 11951-12000, 12001-12050, 12051-12100, 12101-12150, 12151-12200, 12201-12250, 12251-12300, 12301-12350, 12351-12400, 12401-12450, 12451-12500, 12501-12550, 12551-12600, 12601-12650, 12651-12700, 12701-12750, 12751-12800, 12801-12850, 12851-12900, 12901-12950, 12951-13000, 13001-13050, 13051-13100, 13101-13150, 13151-13200, 13201-13250, 13251-13300, 13301-13350, 13351-13400, 13401-

13450, 13451-13500, 13501-13550, 13551-13600, 13601-13650, 13651-13700, 13701-13750, 13751-13800, 13801-13850, 13851-13900, 13901-13950, 13951-14000, 14001-14050, 14051-14100, 14101-14150, 14151-14200, 14201-14250, 14251-14300, 14301-14350, 14351-14400, 14401-14450, 14451-14500, 14501-14550, 14551-14600, 14601-14650, 14651-14700, 14701-14750, 14751-14800, 14801-14850, 14851-14900, 14901-14950, 14951-15000, 15001-15050, 15051-15100, 15101-15150, 15151-15200, 15201-15250, 15251-15300, 15301-15350, 15351-15400, 15401-15450, 15451-15500, 15501-15550, 15551-15600, 15601-15650, 15651-15700, 15701-15750, 15751-15800, 15801-15850, 15851-15900, 15901-15950, 15951-16000, 16001-16050, 16051-16100, 16101-16150, 16151-16200, 16201-16250, 16251-16300, 16301-16350, 16351-16400, 16401-16450, 16451-16500, 16501-16550, 16551-16600, 16601-16650, 16651-16700, 16701-16750, 16751-16800, 16801-16850, 16851-16900, 16901-16950, 16951-17000, 17001-17050, 17051-17100, 17101-17150, 17151-17200, 17201-17250, 17251-17300, 17301-17350, 17351-17400, 17401-17450, 17451-17500, 17501-17550, 17551-17600, 17601-17650, 17651-17700, 17701-17750, 17751-17800, 17801-17850, 17851-17900, 17901-17950, 17951-18000, 18001-18050, 18051-18100, 18101-18150, 18151-18200, 18201-18250, 18251-18300, 18301-18350, 18351-18400, 18401-18450, 18451-18500, 18501-18550, 18551-18600, 18601-18650, 18651-18700, 18701-18750, 18751-18800, 18801-18850, 18851-18900, 18901-18950, 18951-19000, 19001-19050, 19051-19100, 19101-19150, 19151-19200, 19201-19250, 19251-19300, 19301-19350, 19351-19400, 19401-19450, 19451-19500, 19501-19550, 19551-19600, 19601-19650, 19651-19700, 19701-19750, 19751-19800, 19801-19850, 19851-19900, 19901-19950, 19951-20000, 20001-20050, 20051-20100, 20101-20150, 20151-20200, 20201-20250, 20251-20300, 20301-20350, 20351-20400, 20401-20450, 20451-20500, 20501-20550, 20551-20600, 20601-20650, 20651-20700, 20701-20750, 20751-20800, 20801-20850, 20851-20900, 20901-20950, 20951-21000, 21001-21050, 21051-21100, 21101-21150, 21151-21200, 21201-21250, 21251-21300, 21301-21350, 21351-21400, 21401-21450, 21451-21500, 21501-21550, 21551-21600, 21601-21650, 21651-21700, 21701-21750, 21751-21800, 21801-21850, 21851-21900, 21901-21950, 21951-22000, 22001-22050, 22051-22100, 22101-22150, 22151-22200, 22201-22250, 22251-22300, 22301-22350, 22351-22400, 22401-22450, 22451-22500, 22501-22550, 22551-22600, 22601-22650, 22651-22700, 22701-22750, 22751-22800, 22801-22850, 22851-22900, 22901-22950, 22951-23000, 23001-23050, 23051-23100, 23101-23150, 23151-23200, 23201-23250, 23251-23300, 23301-23350, 23351-23400, 23401-23450, 23451-23500, 23501-23550, 23551-23600, 23601-23650, 23751-23700, 23701-23750, 23751-23800, 23801-23850, 23851-23900, 23901-23950, 23951-24000, 24001-24050, 24051-24100, 24101-24150, 24151-24200, 24201-24250, 24251-24300, 24301-24350, 24351-24400, 24401-24450, 24451-

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35600, 35601-35650, 35751-35700, 35701-35750, 35751-35800, 35801-35850, 35851-35900, 35901-35950, 35951-36000, 36001-36050, 36051-36100, 36101-36150, 36151-36200, 36201-36250, 36251-36300, 36301-36350, 36351-36400, 36401-36450, 36451-36500, 36501-36550, 36551-36600, 36601-36650, 36651-36700, 36701-36750, 36751-36800, 36801-36850, 36851-36900, 36901-36950, 36951-37000, 37001-37050, 37051-37100, 37101-37150, 37151-37200, 37201-37250, 37251-37300, 37301-37350, 37351-37400, 37401-37450, 37451-37500, 37501-37550, 37551-37600, 37601-37650, 37651-37700, 37701-37750, 37751-37800, 37801-37850, 37851-37900, 37901-37950, 37951-38000, 38001-38050, 38051-38100, 38101-38150, 38151-38200, 38201-38250, 38251-38300, 38301-38350, 38351-38400, 38401-38450, 38451-38500, 38501-38550, 38551-38600, 38601-38650, 38651-38700, 38701-38750, 38751-38800, 38801-38850, 38851-38900, 38901-38950, 38951-39000, 39001-39050, 39051-39100, 39101-39150, 39151-39200, 39201-39250, 39251-39300, 39301-39350, 39351-39400, 39401-39450, 39451-39500, 39501-39550, 39551-39600, 39601-39650, 39651-39700, 39701-39750, 39751-39800, 39801-39850, 39851-39900, 39901-39950, 39951-40000, 40001-40050, 40051-40100, 40101-40150, 40151-40200, 40201-40250, 40251-40300, 40301-40350, 40351-40400, 40401-40450, 40451-40500, 40501-40550, 40551-40600, 40601-40650, 40651-40700, 40701-40750, 40751-40800, 40801-40850, 40851-40900, 40901-40950, 40951-41000, 41001-41050, 41051-41100, 41101-41150, 41151-41200, 41201-41250, 41251-41300, 41301-41350, 41351-41400, 41401-1450, 31451-31500, 31501-31550, 31551-31600, 31601-31650, 31651-31700, 31701-31750, 41751-41800, 41801-41850, 41851-41900, 41901-41950, 41951-42000, 42001-42050, 42051-42100, 42101-42150, 42151-42200, 42201-42250, 42251-42300, 42301-42350, 42351-42400, 42401-42450, 42451-42500, 42501-42550, 42551-42600, 42601-42650, 42651-42700, 42701-42750, 42751-42800, 42801-42850, 42851-42900, 42901-42950, 42951-43000, 43001-43050, 43051-43100, 43101-43150, 43151-43200, 43201-43250, 43251-43300, 43301-43350, 43351-43400, 43401-43450, 43451-43500, 43501-43550, 43551-43600, 43601-43650, 43751-43700, 43701-43750, 43751-43800, 43801-43850, 43851-43900, 43901-43950, 43951-44000, 44001-44050, 44051-44100, 44101-44150, 44151-44200, 44201-44250, 44251-44300, or 44301-44348, or any combination thereof.

In a further embodiment, the "suitable target segments" identified herein may be employed in a screen for additional compounds that modulate the expression of Notch3. "Modulators" are those compounds that decrease or increase the expression of a nucleic acid molecule encoding Notch3 and which comprise at least an 8-nucleobase portion that is complementary to a suitable target segment. The screening method can comprise, for example, the steps of contacting a target segment of a nucleic acid molecule encoding Notch3 with one or

more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding Notch3. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding Notch3, the modulator may then be employed in further investigative studies of the function of Notch3, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

The suitable target segments of the present invention may be also be combined with their respective complementary compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides. Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processsing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., Nature, 1998, 391, 806-811; Timmons and Fire, Nature 1998, 395, 854; Timmons et al., Gene, 2001, 263, 103-112; Tabara et al., Science, 1998, 282, 430-431; Montgomery et al., Proc. Natl. Acad. Sci. USA, 1998, 95, 15502-15507; Tuschl et al., Genes Dev., 1999, 13, 3191-3197; Elbashir et al., Nature, 2001, 411, 494-498; and Elbashir et al., Genes Dev. 2001, 15, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense strand of the duplex to the target, thereby triggering enzymatic degradation of the target (Tijsterman et al., Science, 2002, 295, 694-697).

The compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the compounds and suitable target segments identified herein in drug discovery efforts to elucidate relationships that exist between Notch3 and a disease state, phenotype, or condition. These methods include, for example, detecting or modulating Notch3 comprising contacting a sample, tissue, cell, or organism with the compounds of the present invention, measuring the nucleic acid or protein level of Notch3 and/or a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample treated with a further compound of the invention. These methods can also be performed in parallel or in combination with other experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

The compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. Furthermore, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary

skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

For use in kits and diagnostics, the compounds of the present invention, either alone or in combination with other compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

As one nonlimiting example, expression patterns within cells or tissues treated with one or more compounds are compared to control cells or tissues not treated with compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds that affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, FEBS Lett., 2000, 480, 17-24; Celis, et al., FEBS Lett., 2000, 480, 2-16), SAGE (serial analysis of gene expression)(Madden, et al., Drug Discov. Today, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, Methods Enzymol., 1999, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., FEBS Lett., 2000, 480, 2-16; Jungblut, et al., Electrophoresis, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., FEBS Lett., 2000, 480, 2-16; Larsson, et al., J. Biotechnol., 2000, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., Anal. Biochem., 2000, 286, 91-98; Larson, et al., Cytometry, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, Curr. Opin. Microbiol., 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., J. Cell Biochem. Suppl., 1998, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, Eur. J. Cancer, 1999, 35, 1895-904) and mass spectrometry methods (To, Comb. Chem. High Throughput Screen, 2000, 3, 235-41).

The compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding Notch3. For example, oligonucleotides that are shown to hybridize with such efficiency and under such conditions as disclosed herein as to be effective Notch3 inhibitors will also be effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding Notch3 and in the

amplification of said nucleic acid molecules for detection or for use in further studies of Notch3. Hybridization of the antisense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid encoding Notch3 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of Notch3 in a sample may also be prepared.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense compounds have been employed as therapeutic moieties in the treatment of disease states in animals, including humans. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that antisense compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and animals, especially humans.

For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of Notch3 is treated by administering antisense compounds in accordance with this invention. For example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of a Notch3 inhibitor. The Notch3 inhibitors of the present invention effectively inhibit the activity of the Notch3 protein or inhibit the expression of the Notch3 protein. In one embodiment, the activity or expression of Notch3 (protein and/or mRNA) in an animal is inhibited by at least 10%, by at least 20%, by at least 25%, by at least 30%, by at least 40%, by at least 50%, by at least 60%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 98%, by at least 99%, or by 100%.

For example, the reduction of the expression of Notch3 may be measured in serum, adipose tissue, liver or any other body fluid, tissue or organ of the animal. In some embodiments, the cells contained within said fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding Notch3 protein and/or the Notch3 protein itself.

The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the compounds and methods of the invention may also be useful prophylactically.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a

phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally favorable. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages (Backbones)

Specific examples of compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821;

5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Modified sugar and internucleoside linkages-Mimetics

In other oligonucleotide mimetics, both the sugar and the internucleoside linkage (i.e. the backbone), of the nucleotide units are replaced with novel groups. The nucleobase units are maintained for hybridization with an appropriate target nucleic acid. One such compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

In some embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH_2-NH-O-CH_2-,

$-CH_2-N(CH_3)-O-CH_2-$ (known as a methylene (methylimino) or MMI backbone), $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ (wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$) of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also suitable are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified sugars

Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particular moieties also include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Another modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. Another modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$, also described in examples hereinbelow.

Other modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. One 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures

include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Another modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene ($-CH_2-)_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Natural and Modified Nucleobases

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Additional nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J.I., ed. John

Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C and are presently suitable base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Conjugates

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed

October 23, 1992, and U.S. Patent 6,287,860, the entire disclosure of which are incorporated herein by reference.

Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928; and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric compounds

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an

oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNAse H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions of endoribonucleases, such as RNAseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. For oligonucleotides, suitable examples of pharmaceutically acceptable salts and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The present invention also includes pharmaceutical compositions and formulations that include the compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug that may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment of the present invention. Emulsions and their uses are well known in the art and are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes that are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

In one embodiment, the present invention employs various penetration enhancers to affect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance

the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Formulations for topical administration include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Bile acids/salts and fatty acids and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Also suitable are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly suitable combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent 6,287,860, which is

incorporated herein in its entirety. Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 09/108,673 (filed July 1, 1998), 09/315,298 (filed May 20, 1999) and 10/071,822, filed February 8, 2002, each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Certain embodiments of the invention provide pharmaceutical compositions containing one or more oligomeric compounds and one or more other chemotherapeutic agents that function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Combinations of antisense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional compounds targeted to a second nucleic acid target. Alternatively, compositions of the invention may contain two or more compounds targeted to different regions of the same

nucleic acid target. Numerous examples of compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same. Each of the references, patents, international publications, GenBank accession numbers, and the like recited in the present application are incoporated herein by reference in its entirety.

EXAMPLES

Example 1: Synthesis of Nucleoside Phosphoramidites

The following compounds, including amidites and their intermediates were prepared as described in US Patent 6,426,220 and published PCT WO 02/36743; 5'-O-Dimethoxytrityl-thymidine intermediate for 5-methyl dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine intermediate for 5-methyl-dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-N4-benzoyl-5-methylcytidine penultimate intermediate for 5-methyl dC amidite, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (5-methyl dC amidite), 2'-Fluorodeoxyadenosine, 2'-Fluorodeoxyguanosine, 2'-Fluorouridine, 2'-Fluorodeoxycytidine, 2'-O-(2-Methoxyethyl)

modified amidites, 2'-O-(2-methoxyethyl)-5-methyluridine intermediate, 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-5-methyluridin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE T amidite), 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate, 5'-O-dimethoxytrityl-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methyl-cytidine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE 5-Me-C amidite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^6-benzoyladenosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE A amdite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-isobutyrylguanosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE G amidite), 2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxy-ethyl) nucleoside amidites, 2'-(Dimethylaminooxyethoxy) nucleoside amidites, 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine , 5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine, 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine , 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine, 5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N dimethylaminooxyethyl]-5-methyluridine, 2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-(Aminooxyethoxy) nucleoside amidites, N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites, 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine, 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine and 5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite.

Example 2: Oligonucleotide and oligonucleoside synthesis

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

Oligonucleotides: Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3,H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1 M NH_4OAc solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Oligonucleosides: Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 3: RNA Synthesis

In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular bulky silyl ethers are used to protect the 5′-hydroxyl in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of 2′ hydroxyl.

Following this procedure for the sequential protection of the 5′-hydroxyl in combination with protection of the 2′-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3′- to 5′-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added, coupling the second base onto the 5′-end of the first nucleoside. The support is washed and any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2′- groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

The 2′-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc.

(Lafayette, CO), is one example of a useful orthoester protecting group which, has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine that not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., J. Am. Chem. Soc., 1998, 120, 11820-11821; Matteucci, M. D. and Caruthers, M. H. J. Am. Chem. Soc., 1981, 103, 3185-3191; Beaucage, S. L. and Caruthers, M. H. Tetrahedron Lett., 1981, 22, 1859-1862; Dahl, B. J., et al., Acta Chem. Scand,. 1990, 44, 639-641; Reddy, M. P., et al., Tetrahedrom Lett., 1994, 25, 4311-4314; Wincott, F. et al., Nucleic Acids Res., 1995, 23, 2677-2684; Griffin, B. E., et al., Tetrahedron, 1967, 23, 2301-2313; and Griffin, B. E., et al., Tetrahedron, 1967, 23, 2315-2331).

RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense compounds can then be annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 µl of each of the complementary strands of RNA oligonucleotides (50 µM RNA oligonucleotide solution) and 15 µl of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at 37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 4: Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap"

segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers."

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in concentrated ammonia (NH_4OH) for 12-16 hr at 55°C. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spetrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry).

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 5: Design and screening of duplexed antisense compounds targeting Notch3

In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target Notch3. The nucleobase sequence of the antisense strand of the duplex comprises at least an 8-nucleobase portion of an oligonucleotide in Table 1. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG (SEQ ID NO:145) and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

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     cgagaggcggacgggaccgTT    (SEQ ID NO:146)    Antisense Strand
     ||||||||||||||||||||
TTgctctccgcctgccctggc          (SEQ ID NO:147)    Complement
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RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 μM. Once diluted, 30 μL of each strand is combined with 15 μL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM magnesium acetate. The final volume is 75 μL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 μM. This solution can be stored frozen (at, for example, -20°C) and freeze-thawed up to 5 times.

Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate Notch3 expression.

When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For cells grown in 96-well plates, wells are washed once with 200 μL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 μL of OPTI-

MEM-1 containing 12 μg/mL LIPOFECTIN (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

Example 6: Oligonucleotide Isolation

After cleavage from the controlled pore glass solid support and deblocking in concentrated ammonium hydroxide at 55°C for 12-16 hours, the oligonucleotides or oligonucleosides are recovered by precipitation out of 1 M NH_4OAc with >3 volumes of ethanol. Synthesized oligonucleotides were analyzed by electrospray mass spectroscopy (molecular weight determination) and by capillary gel electrophoresis and judged to be at least 70% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in the synthesis was determined by the ratio of correct molecular weight relative to the –16 amu product (+/-32 +/-48). For some studies oligonucleotides were purified by HPLC, as described by Chiang et al., J. Biol. Chem. 1991, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7: Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a 96-well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diiso-propyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8: Oligonucleotide Analysis – 96-Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96-well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9: Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, ribonuclease protection assays, or RT-PCR.

T-24 cells: The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #353872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells: The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100

micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells: Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells: Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds: When cells reached 65-75% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 µL OPTI-MEM™-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of oligonucleotide. Cells are treated and data are obtained in triplicate. After 4-7 hours of treatment at 37°C, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is selected from either ISIS 13920 (**TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO:1) which is targeted to human H-ras, or ISIS 18078, (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO:2) which is targeted to human Jun-N-terminal kinase-2 (JNK2). Both controls are 2'-O-methoxyethyl gapmers (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 3, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-H-ras (for ISIS 13920), JNK2 (for ISIS 18078) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of c-H-ras, JNK2 or c-raf mRNA is then utilized as the oligonucleotide screening concentration in

subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments. The concentrations of antisense oligonucleotides used herein are from 50 nM to 300 nM.

Example 10: Analysis of oligonucleotide inhibition of Notch3 expression

Antisense modulation of Notch3 expression can be assayed in a variety of ways known in the art. For example, Notch3 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently favorable. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. A method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of Notch3 can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to Notch3 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

Example 11: Design of phenotypic assays and *in vivo* studies for the use of Notch3 inhibitors

Phenotypic assays

Once Notch3 inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition.

Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of Notch3 in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, Franklin Lakes, NJ; Oncogene

Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies; adipocytes for obesity studies) are treated with Notch3 inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

Analysis of the geneotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the Notch3 inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

In vivo studies

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes humans.

The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study.

To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or Notch3 inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not informed as to whether the medication they are administering is a Notch3 inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

Volunteers receive either the Notch3 inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid molecules encoding Notch3 or Notch3 protein levels in body fluids, tissues or organs compared

to pre-treatment levels. Other measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/great) and number and type of previous treatment regimens for the indicated disease or condition.

Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers with certain characteristics are equally distributed for placebo and Notch3 inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers treated with the Notch3 inhibitor show positive trends in their disease state or condition index at the conclusion of the study.

Example 12: RNA Isolation

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., (Clin. Chem., 1996, 42, 1758-1764). Other methods for poly(A)+ mRNA isolation are routine in the art. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C, was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed

with 200 μL cold PBS. 150 μL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 μL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 μL of Buffer RW1 was added to each well of the RNEASY 96™ plate and incubated for 15 minutes and the vacuum was again applied for 1 minute. An additional 500 μL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 140 μL of RNAse free water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13: Real-time Quantitative PCR Analysis of Notch3 mRNA Levels

Quantitation of Notch3 mRNA levels was accomplished by real-time quantitative PCR using the ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system that allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is

quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen Corporation, (Carlsbad, CA). RT-PCR reactions were carried out by adding 20 μL PCR cocktail (2.5x PCR buffer minus $MgCl_2$, 6.6 mM $MgCl_2$, 375 μM each of dATP, dCTP, dCTP and dGTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNAse inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96-well plates containing 30 μL total RNA solution (20-200 ng). The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by

real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent (Molecular Probes, Inc. Eugene, OR). Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374).

In this assay, 170 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 µL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 485nm and emission at 530nm.

Probes and primers to human Notch3 were designed to hybridize to a human Notch3 sequence, using published sequence information (the complement of residues 118831-163178 of GenBank accession number NT_011290.3, representing a genomic sequence of Notch3, incorporated herein as SEQ ID NO: 4). For human Notch3 the PCR primers were:

forward primer: TCACCATGCCGTAACGGG (SEQ ID NO: 5)

reverse primer: TCGGTGTCCTGGACAGTCG (SEQ ID NO: 6)

and the PCR probe was:

FAM-CTTCCTGGGTTTGAGGGTCAGAATTGTG-TAMRA (SEQ ID NO: 7)

where FAM is the fluorescent dye and TAMRA is the quencher dye. For human GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC(SEQ ID NO:8)

reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:9)

and the PCR probe was:

5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO: 10)

where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Example 14: Northern blot analysis of Notch3 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc,

La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human Notch3, a human Notch3 specific probe was prepared by PCR using the forward primer TCACCATGCCGTAACGGG (SEQ ID NO: 5) and the reverse primer TCGGTGTCCTGGACAGTCG (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15: Antisense inhibition of human Notch3 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of antisense compounds were designed to target different regions of the human Notch3 RNA, using published sequences (the complement of residues 118831-163178 of GenBank accession number NT_011290.3, representing a genomic sequence of Notch3, incorporated herein as SEQ ID NO: 4; GenBank accession number NM_000435.1, incorporated herein as SEQ ID NO:11). The compounds are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human Notch3 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments in which A549 cells were treated with the oligonucleotides of the present invention. The positive control for each datapoint is identified in the table by sequence ID number. If present, "N.D." indicates "no data."

Table 1

Inhibition of human Notch3 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
226740	Coding	4	1661	cccgctagcagcagcagcag	69	12	1
226741	Coding	4	4930	caacgacctccatttgcaca	59	13	1
226742	Coding	4	4935	gggtgcaacgacctccattt	17	14	1
226743	Coding	4	10042	gccaccactgaactctggca	77	15	1
226744	Coding	4	10638	ggacagtcgtccacgttcac	83	16	1
226745	Coding	4	10708	aggagggcactggcagttat	55	17	1
226746	Coding	4	10947	atattctgactgcagctctc	79	18	1
226747	Coding	11	1105	cacaggaggccagtcttgcc	51	19	1
226748	Coding	4	13104	tttgtgtcacagatagcatc	70	20	1
226749	Coding	4	13385	gtctcacagcgaggtccagt	92	21	1
226750	Coding	11	1450	gttcctgtgaagcctgccat	86	22	1
226751	Coding	4	14216	tgcagctgaagccattgact	78	23	1
226752	Coding	4	15268	ccacctggctctcgcagcgt	82	24	1
226753	Coding	4	15336	gcagaggtacttgtccacca	78	25	1
226754	Coding	4	15341	cagcggcagaggtacttgtc	56	26	1
226755	Coding	11	1912	tcgcagttcacacctgtggt	77	27	1
226756	Coding	4	15553	cggttgatgccatcacggca	68	28	1
226757	Coding	4	16814	cactcattgatctccacgtt	59	29	1
226758	Coding	4	16862	ttttccccatccacacagga	34	30	1
226759	Coding	4	18037	tccacatcctgctggcatcg	46	31	1
226760	Coding	4	20876	gtcgggcaggtcctgttcgc	65	32	1
226761	Coding	4	21386	ggcagtgggctcctgtgtag	66	33	1
226762	Coding	4	22235	cgcctgacacagctgctcca	82	34	1
226763	Coding	4	22284	ctgggcacacgcagtagtgg	55	35	1
226764	Coding	4	22372	atatagccacggcaggtccc	69	36	1
226765	Coding	11	3396	caggaagacactcacacatg	59	37	1
226766	Coding	4	23287	gaaaccacccaccaggtcca	77	38	1
226767	Coding	4	23385	cagtcccgggtgtgtgccgc	52	39	1
226768	Coding	4	23588	cggcactggcctccatgctg	98	40	1
226769	Coding	4	23627	caggtgaaggtcagcccacc	26	41	1
226770	Coding	4	23632	agtgacaggtgaaggtcagc	59	42	1
226771	Coding	4	24425	tgcagctcccggcaggagcg	51	43	1
226772	Coding	4	24430	ggcactgcagctcccggcag	65	44	1
226773	Coding	4	24764	cagcctgggctgttgcactc	69	45	1
226774	Coding	4	24861	gcagcggctgttgttgaaga	70	46	1
226775	Coding	4	28093	gtggtcggcgcagtacttct	54	47	1
226776	Coding	4	28098	gcaaagtggtcggcgcagta	77	48	1
226777	Coding	4	32045	aaccagagggtgctgtgctc	63	49	1
226778	Coding	11	5188	cccttggccatgttcttcat	83	50	1
226779	Intron: Exon Junction	4	32312	tctcaccttggccatgttc	86	51	1
226780	Coding	4	32342	tccagtctgtggccacctcc	90	52	1
226781	Coding	11	5275	atgcctggctcctctacctt	78	53	1
226782	Coding	4	35155	agtgccatggctggtgccac	78	54	1
226783	Coding	4	36465	ctagctgatgtgtcatctgc	71	55	1
226784	Coding	4	36603	gtgtctgccccagcatccag	82	56	1
226785	Coding	4	37060	gcgatgagctcttccaccat	69	57	1
226786	Coding	4	37065	ggctggcgatgagctcttcc	28	58	1
226787	Coding	4	40803	gcttggcagcctcatagctg	36	59	1
226788	Coding	4	40808	cagcagcttggcagcctcat	80	60	1

226789	Coding	4	40920	cactgggttgatccagcaag	65	61	1
226790	Coding	4	41240	cactgcagtggcagtggcag	87	62	1
226791	Coding	4	41548	acccgcaggaagcgggcctt	47	63	1
226792	Coding	4	41553	tgggaacccgcaggaagcgg	6	64	1
226793	Coding	4	41637	cggaccagtctgagagggag	51	65	1
226794	Stop Codon	4	41806	gcgtctcaggccaacacttg	76	66	1
226795	3'UTR	4	41826	caagatctaagaactgacga	75	67	1
226796	3'UTR	4	41936	caaggcaaggatgcaggagg	72	68	1
226797	3'UTR	4	42120	ttcatgtcagagtgtaagga	64	69	1
226798	3'UTR	4	42225	catatatatattttgtaaaa	21	70	1
226799	3'UTR	4	42280	cacagactcagccccacggg	67	71	1
226800	3'UTR	4	42302	atccagcttggccgaatggg	69	72	1
226801	3'UTR	4	42363	tacctgggtcgtgtactcgg	66	73	1
226802	3'UTR	4	42401	gccccagtgggtgcgcccaa	69	74	1
226803	3'UTR	4	42465	tggaatgcagtgaagtgagg	83	75	1
226804	3'UTR	4	42505	gtgggcccttccccagcaag	67	76	1
226805	3'UTR	4	42557	agttcccaaagggagatggc	71	77	1
226806	3'UTR	11	7852	tcccacatttacagggacat	63	78	1
226807	3'UTR	4	42651	tcaggcagctcctcttcttg	81	79	1
226808	3'UTR	4	42682	ccagaggattaccaggaaga	67	80	1
226809	3'UTR	4	42713	aaaaatacctctattctgcc	24	81	1
226810	Intron	4	3944	ggctggtggatcacctgagg	56	82	1
226811	Intron	4	15976	ctccgcctcctgaggtcaag	71	83	1
226812	Intron	4	20601	gggatatgccttggattgag	28	84	1
226813	Intron: Exon Junction	4	24948	atgggctcacttgcaagtgc	39	85	1
226814	Intron: Exon Junction	4	28405	ggtcactcacccgatcacct	53	86	1
226815	Intron: Exon Junction	4	35224	tgtcactaacctgggccacg	83	87	1
226816	Intron: Exon Junction	4	36957	ggatgagaatctaggacaga	14	88	1
226817	Intron	4	40290	ggttttactacgttggccag	78	89	1

As shown in Table 1, SEQ ID NOs 12, 13, 15, 16, 18, 20, 21, 22, 23, 24, 25, 27, 28, 29, 32, 33, 34, 36, 37, 38, 40, 42, 44, 45, 46, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 60, 61, 62, 66, 67, 68, 69, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 83, 87 and 89 demonstrated at least 59% inhibition of human Notch3 expression in this assay and are therefore suitable. SEQ ID NOs:21, 51 and 52 showed the best results. The target regions to which these sequences are complementary are herein referred to as "suitable target segments" and are therefore suitable for targeting by compounds of the present invention. These suitable target segments are shown in Table 2. The sequences represent the reverse complement of the suitable compounds shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 2 is the species in which each of the suitable target segments was found.

Table 2

Sequence and position of suitable target regions identified in Notch3.

SITEID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
143392	4	1661	ctgctgctgctgctagcggg	12	*H. sapiens*	90
143393	4	4930	tgtgcaaatggaggtcgttg	13	*H. sapiens*	91
143395	4	10042	tgccagagttcagtggtggc	15	*H. sapiens*	92
143396	4	10638	gtgaacgtggacgactgtcc	16	*H. sapiens*	93
143398	4	10947	gagagctgcagtcagaatat	18	*H. sapiens*	94
143400	4	13104	gatgctatctgtgacacaaa	20	*H. sapiens*	95
143401	4	13385	actggacctcgctgtgagac	21	*H. sapiens*	96
143402	11	1450	atggcaggcttcacaggaac	22	*H. sapiens*	97
143403	4	14216	agtcaatggcttcagctgca	23	*H. sapiens*	98
143404	4	15268	acgctgcgagagccaggtgg	24	*H. sapiens*	99
143405	4	15336	tggtggacaagtacctctgc	25	*H. sapiens*	100
143407	11	1912	accacaggtgtgaactgcga	27	*H. sapiens*	101
143408	4	15553	tgccgtgatggcatcaaccg	28	*H. sapiens*	102
143409	4	16814	aacgtggagatcaatgagtg	29	*H. sapiens*	103
143412	4	20876	gcgaacaggacctgcccgac	32	*H. sapiens*	104
143413	4	21386	ctacacaggagcccactgcc	33	*H. sapiens*	105
143414	4	22235	tggagcagctgtgtcaggcg	34	*H. sapiens*	106
143416	4	22372	gggacctgccgtggctatat	36	*H. sapiens*	107
143417	11	3396	catgtgtgagtgtcttcctg	37	*H. sapiens*	108
143418	4	23287	tggacctggtgggtggtttc	38	*H. sapiens*	109
143420	4	23588	cagcatggaggccagtgccg	40	*H. sapiens*	110
143422	4	23632	gctgaccttcacctgtcact	42	*H. sapiens*	111
143424	4	24430	ctgccgggagctgcagtgcc	44	*H. sapiens*	112
143425	4	24764	gagtgcaacagcccaggctg	45	*H. sapiens*	113
143426	4	24861	tcttcaacaacagccgctgc	46	*H. sapiens*	114
143428	4	28098	tactgcgccgaccactttgc	48	*H. sapiens*	115
143429	4	32045	gagcacagcaccctctggtt	49	*H. sapiens*	116
143430	11	5188	atgaagaacatggccaaggg	50	*H. sapiens*	117
143431	4	32312	gaacatggccaagggtgaga	51	*H. sapiens*	118
143432	4	32342	ggaggtggccacagactgga	52	*H. sapiens*	119
143433	11	5275	aaggtagaggagccaggcat	53	*H. sapiens*	120
143434	4	35155	gtggcaccagccatggcact	54	*H. sapiens*	121
143435	4	36465	gcagatgacacatcagctag	55	*H. sapiens*	122
143436	4	36603	ctggatgctggggcagacac	56	*H. sapiens*	123
143437	4	37060	atggtggaagagctcatcgc	57	*H. sapiens*	124
143440	4	40808	atgaggctgccaagctgctg	60	*H. sapiens*	125
143441	4	40920	cttgctggatcaacccagtg	61	*H. sapiens*	126
143442	4	41240	ctgccactgccactgcagtg	62	*H. sapiens*	127
143446	4	41806	caagtgttggcctgagacgc	66	*H. sapiens*	128
143447	4	41826	tcgtcagttcttagatcttg	67	*H. sapiens*	129
143448	4	41936	cctcctgcatccttgccttg	68	*H. sapiens*	130
143449	4	42120	tccttacactctgacatgaa	69	*H. sapiens*	131
143451	4	42280	cccgtggggctgagtctgtg	71	*H. sapiens*	132
143452	4	42302	cccattcggccaagctggat	72	*H. sapiens*	133
143453	4	42363	ccgagtacacgacccaggta	73	*H. sapiens*	134
143454	4	42401	ttgggcgcacccactggggc	74	*H. sapiens*	135
143455	4	42465	cctcacttcactgcattcca	75	*H. sapiens*	136
143456	4	42505	cttgctggggaagggcccac	76	*H. sapiens*	137
143457	4	42557	gccatctccctttgggaact	77	*H. sapiens*	138
143458	11	7852	atgtccctgtaaatgtggga	78	*H. sapiens*	139
143459	4	42651	caagaagaggagctgcctga	79	*H. sapiens*	140
143460	4	42682	tcttcctggtaatcctctgg	80	*H. sapiens*	141
143463	4	15976	cttgacctcaggaggcggag	83	*H. sapiens*	142

143467	4	35224	cgtggcccaggttagtgaca	87	*H. sapiens*	143
143469	4	40290	ctggccaacgtagtaaaacc	89	*H. sapiens*	144

As these "suitable target segments" have been found by experimentation to be open to, and accessible for, hybridization with the compounds of the present invention, one of skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these suitable target segments and consequently inhibit the expression of Notch3.

According to the present invention, antisense compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other short oligomeric compounds that hybridize to at least a portion of the target nucleic acid.

Example 16: Western blot analysis of Notch3 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to Notch3 is used, with a radiolabeled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

1. A compound 8 to 80 nucleobases in length targeted to a nucleic acid molecule encoding Notch3, wherein the compound specifically hybridizes with the nucleic acid molecule encoding Notch3 (SEQ ID NO: 4) and inhibits the expression of Notch3.

2. The compound of claim 1 comprising 12 to 50 nucleobases in length.

3. The compound of claim 2 comprising 15 to 30 nucleobases in length.

4. The compound of claim 1 comprising an oligonucleotide.

5. The compound of claim 4 comprising an antisense oligonucleotide.

6. The compound of claim 4 comprising a DNA oligonucleotide.

7. The compound of claim 4 comprising an RNA oligonucleotide.

8. The compound of claim 4 comprising a chimeric oligonucleotide.

9. The compound of claim 4 wherein at least a portion of the compound hybridizes with RNA to form an oligonucleotide-RNA duplex.

10. The compound of claim 1 having at least 70% complementarity with a nucleic acid molecule encoding Notch3 (SEQ ID NO: 4) the compound specifically hybridizing to and inhibiting the expression of Notch3.

11. The compound of claim 1 having at least 80% complementarity with a nucleic acid molecule encoding Notch3 (SEQ ID NO: 4) the compound specifically hybridizing to and inhibiting the expression of Notch3.

12. The compound of claim 1 having at least 90% complementarity with a nucleic acid molecule encoding Notch3 (SEQ ID NO: 4) the compound specifically hybridizing to and inhibiting the expression of Notch3.

13. The compound of claim 1 having at least 95% complementarity with a nucleic acid molecule encoding Notch3 (SEQ ID NO: 4) the compound specifically hybridizing to and inhibiting the expression of Notch3.

14. The compound of claim 1 having at least one modified internucleoside linkage, sugar moiety, or nucleobase.

15. The compound of claim 1 having at least one 2'-O-methoxyethyl sugar moiety.

16. The compound of claim 1 having at least one phosphorothioate internucleoside linkage.

17. The compound of claim 1 having at least one 5-methylcytosine.

18. The compound of claim 1 wherein the compound specifically hybridizes to the 5' untranslated region, the start codon region, the coding region, the stop codon region, or the 3' untranslated region of the nucleic acid molecule encoding Notch3.

19. The compound of claim 1 wherein the compound specifically hybridizes to the 5' untranslated region.

20. The compound of claim 1 wherein the compound specifically hybridizes to the start codon region.

21. The compound of claim 1 wherein the compound specifically hybridizes to the coding region.

22. The compound of claim 1 wherein the compound specifically hybridizes to the stop codon region.

23. The compound of claim 1 wherein the compound specifically hybridizes to the 3' untranslated region.

24. The compound of claim 1 wherein the compound comprises SEQ ID NO:21, 51, 52, 12, 13, 15, 16, 18, 20, 22, 23, 24, 25, 27, 28, 29, 32, 33, 34, 36, 37, 38, 40, 42, 44, 45, 46, 48, 49, 50, 53, 54, 55, 56, 57, 60, 61, 62, 66, 67, 68, 69, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 83, 87, or 89.

25. The compound of claim 1 wherein the compound comprises SEQ ID NO:21, 51, or 52.

26. A method of inhibiting the expression of Notch3 in cells or tissues comprising contacting the cells or tissues with the compound of claim 1 so that expression of Notch3 is inhibited.

27. A method of screening for a modulator of Notch3, the method comprising the steps of:

contacting a suitable target segment of a nucleic acid molecule encoding Notch3 with one or more candidate modulators of Notch3; and

identifying one or more modulators of Notch3 expression which modulate the expression of Notch3.

28. The method of claim 27 wherein the modulator of Notch3 expression comprises an oligonucleotide, an antisense oligonucleotide, a DNA oligonucleotide, an RNA oligonucleotide, an RNA oligonucleotide having at least a portion of said RNA oligonucleotide capable of hybridizing with RNA to form an oligonucleotide-RNA duplex, or a chimeric oligonucleotide.

29. A diagnostic method for identifying a disease state comprising identifying the presence of Notch3 in a sample using at least one of the primers comprising SEQ ID NOs: 5 or 6, or the probe comprising SEQ ID NO: 7.

30. A kit or assay device comprising the compound of claim 1.

31. A method of treating an animal having a disease or condition associated with Notch3 comprising administering to the animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of Notch3 is inhibited.

32. The method of claim 31 wherein the disease or condition is a hyperproliferative disorder.

SEQUENCE LISTING

<110> Susan M. Freier
Kenneth W. Dobie

<120> MODULATION OF NOTCH3 EXPRESSION

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Met Gly Pro Gly Ala Arg Gly Arg Arg Arg Arg
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Arg Arg Pro Met Ser Pro Pro Pro Pro Pro Pro Val Arg Ala Leu
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Pro Leu Leu Leu Leu Leu Ala Gly Pro Gly Ala Ala Ala Pro Pro Cys
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Leu Asp Gly Ser Pro Cys Ala Asn Gly Gly Arg Cys Thr Gln Leu Pro
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Ser Arg Glu Ala Ala Cys Leu Cys Pro Pro Gly Trp Val Gly Glu Arg
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Cys Gln Leu Glu Asp Pro Cys His Ser Gly Pro Cys Ala Gly Arg Gly
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Val Cys Gln Ser Ser Val Val Ala Gly Thr Ala Arg Phe Ser Cys Arg
95 100 105

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Cys Pro Arg Gly Phe Arg Gly Pro Asp Cys Ser Leu Pro Asp Pro Cys
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Leu Ser Ser Pro Cys Ala His Gly Ala Arg Cys Ser Val Gly Pro Asp
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Gly Arg Phe Leu Cys Ser Cys Pro Pro Gly Tyr Gln Gly Arg Ser Cys
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cga agc gac gtg gat gag tgc cgg gtg ggt gag ccc tgc cgc cat ggt     591
Arg Ser Asp Val Asp Glu Cys Arg Val Gly Glu Pro Cys Arg His Gly
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ggc acc tgc ctc aac aca cct ggc tcc ttc cgc tgc cag tgt cca gct     639
Gly Thr Cys Leu Asn Thr Pro Gly Ser Phe Arg Cys Gln Cys Pro Ala
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ggc tac aca ggg cca cta tgt gag aac ccc gcg gtg ccc tgt gcg ccc     687
Gly Tyr Thr Gly Pro Leu Cys Glu Asn Pro Ala Val Pro Cys Ala Pro
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Ser Pro Cys Arg Asn Gly Gly Thr Cys Arg Gln Ser Gly Asp Leu Thr
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tac gac tgt gcc tgt ctt cct ggg ttt gag ggt cag aat tgt gaa gtg     783
Tyr Asp Cys Ala Cys Leu Pro Gly Phe Glu Gly Gln Asn Cys Glu Val
220                 225                 230                 235

aac gtg gac gac tgt cca gga cac cga tgt ctc aat ggg ggg aca tgc     831
Asn Val Asp Asp Cys Pro Gly His Arg Cys Leu Asn Gly Gly Thr Cys
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gtg gat ggc gtc aac acc tat aac tgc cag tgc cct cct gag tgg aca     879
Val Asp Gly Val Asn Thr Tyr Asn Cys Gln Cys Pro Pro Glu Trp Thr
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ggc cag ttc tgc acg gag gac gtg gat gag tgt cag ctg cag ccc aac     927
Gly Gln Phe Cys Thr Glu Asp Val Asp Glu Cys Gln Leu Gln Pro Asn
        270                 275                 280

gcc tgc cac aat ggg ggt acc tgc ttc aac acg ctg ggt ggc cac agc     975
Ala Cys His Asn Gly Gly Thr Cys Phe Asn Thr Leu Gly Gly His Ser
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tgc gtg tgt gtc aat ggc tgg aca ggt gag agc tgc agt cag aat atc    1023
Cys Val Cys Val Asn Gly Trp Thr Gly Glu Ser Cys Ser Gln Asn Ile
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gat gac tgt gcc aca gcc gtg tgc ttc cat ggg gcc acc tgc cat gac    1071
Asp Asp Cys Ala Thr Ala Val Cys Phe His Gly Ala Thr Cys His Asp
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cgc gtg gct tct ttc tac tgt gcc tgc ccc atg ggc aag act ggc ctc    1119
Arg Val Ala Ser Phe Tyr Cys Ala Cys Pro Met Gly Lys Thr Gly Leu
            335                 340                 345

ctg tgt cac ctg gat gac gcc tgt gtc agc aac ccc tgc cac gag gat    1167
Leu Cys His Leu Asp Asp Ala Cys Val Ser Asn Pro Cys His Glu Asp
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gct atc tgt gac aca aat ccg gtg aac ggc cgg gcc att tgc acc tgt    1215
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Ala Ile Cys Asp Thr Asn Pro Val Asn Gly Arg Ala Ile Cys Thr Cys
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Pro Pro Gly Phe Thr Gly Gly Ala Cys Asp Gln Asp Val Asp Glu Cys
380                 385                 390                 395

tct atc ggc gcc aac ccc tgc gag cac ttg ggc agg tgc gtg aac acg    1311
Ser Ile Gly Ala Asn Pro Cys Glu His Leu Gly Arg Cys Val Asn Thr
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cag ggc tcc ttc ctg tgc cag tgc ggt cgt ggc tac act gga cct cgc    1359
Gln Gly Ser Phe Leu Cys Gln Cys Gly Arg Gly Tyr Thr Gly Pro Arg
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tgt gag acc gat gtc aac gag tgt ctg tcg ggg ccc tgc cga aac cag    1407
Cys Glu Thr Asp Val Asn Glu Cys Leu Ser Gly Pro Cys Arg Asn Gln
        430                 435                 440

gcc acg tgc ctc gac cgc ata ggc cag ttc acc tgt atc tgt atg gca    1455
Ala Thr Cys Leu Asp Arg Ile Gly Gln Phe Thr Cys Ile Cys Met Ala
    445                 450                 455

ggc ttc aca gga acc tat tgc gag gtg gac att gac gag tgt cag agt    1503
Gly Phe Thr Gly Thr Tyr Cys Glu Val Asp Ile Asp Glu Cys Gln Ser
460                 465                 470                 475

agc ccc tgt gtc aac ggt ggg gtc tgc aag gac cga gtc aat ggc ttc    1551
Ser Pro Cys Val Asn Gly Gly Val Cys Lys Asp Arg Val Asn Gly Phe
                480                 485                 490

agc tgc acc tgc ccc tcg ggc ttc agc ggc tcc acg tgt cag ctg gac    1599
Ser Cys Thr Cys Pro Ser Gly Phe Ser Gly Ser Thr Cys Gln Leu Asp
            495                 500                 505

gtg gac gaa tgc gcc agc acg ccc tgc agg aat ggc gcc aaa tgc gtg    1647
Val Asp Glu Cys Ala Ser Thr Pro Cys Arg Asn Gly Ala Lys Cys Val
        510                 515                 520

gac cag ccc gat ggc tac gag tgc cgc tgt gcc gag ggc ttt gag ggc    1695
Asp Gln Pro Asp Gly Tyr Glu Cys Arg Cys Ala Glu Gly Phe Glu Gly
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acg ctg tgt gat cgc aac gtg gac gac tgc tcc cct gac cca tgc cac    1743
Thr Leu Cys Asp Arg Asn Val Asp Asp Cys Ser Pro Asp Pro Cys His
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cat ggt cgc tgc gtg gat ggc atc gcc agc ttc tca tgt gcc tgt gct    1791
His Gly Arg Cys Val Asp Gly Ile Ala Ser Phe Ser Cys Ala Cys Ala
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cct ggc tac acg ggc aca cgc tgc gag agc cag gtg gac gaa tgc cgc    1839
Pro Gly Tyr Thr Gly Thr Arg Cys Glu Ser Gln Val Asp Glu Cys Arg
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Ser Gln Pro Cys Arg His Gly Gly Lys Cys Leu Asp Leu Val Asp Lys
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Tyr Leu Cys Arg Cys Pro Ser Gly Thr Thr Gly Val Asn Cys Glu Val
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aac att gac gac tgt gcc agc aac ccc tgc acc ttt gga gtc tgc cgt    1983
Asn Ile Asp Asp Cys Ala Ser Asn Pro Cys Thr Phe Gly Val Cys Arg
620                 625                 630                 635

gat ggc atc aac cgc tac gac tgt gtc tgc caa cct ggc ttc aca ggg    2031
Asp Gly Ile Asn Arg Tyr Asp Cys Val Cys Gln Pro Gly Phe Thr Gly
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ccc ctt tgt aac gtg gag atc aat gag tgt gct tcc agc cca tgc ggc    2079
Pro Leu Cys Asn Val Glu Ile Asn Glu Cys Ala Ser Ser Pro Cys Gly
            655                 660                 665

gag gga ggt tcc tgt gtg gat ggg gaa aat ggc ttc cgc tgc ctc tgc    2127
Glu Gly Gly Ser Cys Val Asp Gly Glu Asn Gly Phe Arg Cys Leu Cys
        670                 675                 680

ccg cct ggc tcc ttg ccc cca ctc tgc ctc ccc ccg agc cat ccc tgt    2175
Pro Pro Gly Ser Leu Pro Pro Leu Cys Leu Pro Pro Ser His Pro Cys
    685                 690                 695

gcc cat gag ccc tgc agt cac ggc atc tgc tat gat gca cct ggc ggg    2223
Ala His Glu Pro Cys Ser His Gly Ile Cys Tyr Asp Ala Pro Gly Gly
700                 705                 710                 715

ttc cgc tgt gtg tgt gag cct ggc tgg agt ggc ccc cgc tgc agc cag    2271
Phe Arg Cys Val Cys Glu Pro Gly Trp Ser Gly Pro Arg Cys Ser Gln
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agc ctg gcc cga gac gcc tgt gag tcc cag ccg tgc agg gcc ggt ggg    2319
Ser Leu Ala Arg Asp Ala Cys Glu Ser Gln Pro Cys Arg Ala Gly Gly
            735                 740                 745

aca tgc agc agc gat gga atg ggt ttc cac tgc acc tgc ccg cct ggt    2367
Thr Cys Ser Ser Asp Gly Met Gly Phe His Cys Thr Cys Pro Pro Gly
        750                 755                 760

gtc cag gga cgt cag tgt gaa ctc ctc tcc ccc tgc acc ccg aac ccc    2415
Val Gln Gly Arg Gln Cys Glu Leu Leu Ser Pro Cys Thr Pro Asn Pro
    765                 770                 775

tgt gag cat ggg ggc cgc tgc gag tct gcc cct ggc cag ctg cct gtc    2463
Cys Glu His Gly Gly Arg Cys Glu Ser Ala Pro Gly Gln Leu Pro Val
780                 785                 790                 795

tgc tcc tgc ccc cag ggc tgg caa ggc cca cga tgc cag cag gat gtg    2511
Cys Ser Cys Pro Gln Gly Trp Gln Gly Pro Arg Cys Gln Gln Asp Val
                800                 805                 810

gac gag tgt gct ggc ccc gca ccc tgt ggc cct cat ggt atc tgc acc    2559
Asp Glu Cys Ala Gly Pro Ala Pro Cys Gly Pro His Gly Ile Cys Thr
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aac ctg gca ggg agt ttc agc tgc acc tgc cat gga ggg tac act ggc    2607
Asn Leu Ala Gly Ser Phe Ser Cys Thr Cys His Gly Gly Tyr Thr Gly
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cct tcc tgt gat cag gac atc aat gac tgt gac ccc aac cca tgc ctg    2655
Pro Ser Cys Asp Gln Asp Ile Asn Asp Cys Asp Pro Asn Pro Cys Leu
    845                 850                 855

aac ggt ggc tcg tgc caa gac ggc gtg ggc tcc ttt tcc tgc tcc tgc    2703
Asn Gly Gly Ser Cys Gln Asp Gly Val Gly Ser Phe Ser Cys Ser Cys
860                 865                 870                 875

ctc cct ggt ttc gcc ggc cca cga tgc gcc cgc gat gtg gat gag tgc    2751
Leu Pro Gly Phe Ala Gly Pro Arg Cys Ala Arg Asp Val Asp Glu Cys
                880                 885                 890

ctg agc aac ccc tgc ggc ccg ggc acc tgt acc gac cac gtg gcc tcc    2799
Leu Ser Asn Pro Cys Gly Pro Gly Thr Cys Thr Asp His Val Ala Ser
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ttc acc tgc acc tgc ccg ccg ggc tac gga ggc ttc cac tgc gaa cag    2847
Phe Thr Cys Thr Cys Pro Pro Gly Tyr Gly Gly Phe His Cys Glu Gln
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gac ctg ccc gac tgc agc ccc agc tcc tgc ttc aat ggc ggg acc tgt    2895
Asp Leu Pro Asp Cys Ser Pro Ser Ser Cys Phe Asn Gly Gly Thr Cys
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gtg gac ggc gtg aac tcg ttc agc tgc ctg tgc cgt ccc ggc tac aca    2943
Val Asp Gly Val Asn Ser Phe Ser Cys Leu Cys Arg Pro Gly Tyr Thr
940                 945                 950                 955

gga gcc cac tgc caa cat gag gca gac ccc tgc ctc tcg cgg ccc tgc    2991
Gly Ala His Cys Gln His Glu Ala Asp Pro Cys Leu Ser Arg Pro Cys
                960                 965                 970

cta cac ggg ggc gtc tgc agc gcc gcc cac cct ggc ttc cgc tgc acc    3039
Leu His Gly Gly Val Cys Ser Ala Ala His Pro Gly Phe Arg Cys Thr
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tgc ctc gag agc ttc acg ggc ccg cag tgc cag acg ctg gtg gat tgg    3087
Cys Leu Glu Ser Phe Thr Gly Pro Gln Cys Gln Thr Leu Val Asp Trp
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tgc agc cgc cag cct tgt caa aac ggg ggt cgc tgc gtc cag act ggg    3135
Cys Ser Arg Gln Pro Cys Gln Asn Gly Gly Arg Cys Val Gln Thr Gly
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gcc tat tgc ctt tgt ccc cct gga tgg agc gga cgc ctc tgt gac atc    3183
Ala Tyr Cys Leu Cys Pro Pro Gly Trp Ser Gly Arg Leu Cys Asp Ile
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cga agc ttg ccc tgc agg gag gcc gca gcc cag atc ggg gtg cgg ctg    3231
Arg Ser Leu Pro Cys Arg Glu Ala Ala Ala Gln Ile Gly Val Arg Leu
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cac tac tgc gtg tgc cca gag ggc cgt act ggt agc cac tgt gag cag    3327
His Tyr Cys Val Cys Pro Glu Gly Arg Thr Gly Ser His Cys Glu Gln
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gag gtg gac ccc tgc ttg gcc cag ccc tgc cag cat ggg ggg acc tgc    3375
Glu Val Asp Pro Cys Leu Ala Gln Pro Cys Gln His Gly Gly Thr Cys
    1085                1090                1095

cgt ggc tat atg ggg ggc tac atg tgt gag tgt ctt cct ggc tac aat    3423
Arg Gly Tyr Met Gly Gly Tyr Met Cys Glu Cys Leu Pro Gly Tyr Asn
1100                1105                1110                1115

ggt gat aac tgt gag gac gac gtg gac gag tgt gcc tcc cag ccc tgc    3471
Gly Asp Asn Cys Glu Asp Asp Val Asp Glu Cys Ala Ser Gln Pro Cys
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Gln His Gly Gly Ser Cys Ile Asp Leu Val Ala Arg Tyr Leu Cys Ser
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tgt ccc cca gga acg ctg ggg gtg ctc tgc gag att aat gag gat gac    3567
Cys Pro Pro Gly Thr Leu Gly Val Leu Cys Glu Ile Asn Glu Asp Asp
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tgc ggc cca ggc cca ccg ctg gac tca ggg ccc cgg tgc cta cac aat    3615
Cys Gly Pro Gly Pro Pro Leu Asp Ser Gly Pro Arg Cys Leu His Asn
    1165                1170                1175

ggc acc tgc gtg gac ctg gtg ggt ggt ttc cgc tgc acc tgt ccc cca    3663
Gly Thr Cys Val Asp Leu Val Gly Gly Phe Arg Cys Thr Cys Pro Pro
1180                1185                1190                1195

gga tac act ggt ttg cgc tgc gag gca gac atc aat gag tgt cgc tca    3711
Gly Tyr Thr Gly Leu Arg Cys Glu Ala Asp Ile Asn Glu Cys Arg Ser
                1200                1205                1210

ggt gcc tgc cac gcg gca cac acc cgg gac tgc ctg cag gac cca ggc    3759
Gly Ala Cys His Ala Ala His Thr Arg Asp Cys Leu Gln Asp Pro Gly
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gga ggt ttc cgt tgc ctt tgt cat gct ggc ttc tca ggt cct cgc tgt    3807
Gly Gly Phe Arg Cys Leu Cys His Ala Gly Phe Ser Gly Pro Arg Cys
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cag act gtc ctg tct ccc tgc gag tcc cag cca tgc cag cat gga ggc    3855
Gln Thr Val Leu Ser Pro Cys Glu Ser Gln Pro Cys Gln His Gly Gly
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cag tgc cgt cct agc ccg ggt cct ggg ggt ggg ctg acc ttc acc tgt    3903
Gln Cys Arg Pro Ser Pro Gly Pro Gly Gly Gly Leu Thr Phe Thr Cys
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cac tgt gcc cag ccg ttc tgg ggt ccg cgt tgc gag cgg gtg gcg cgc    3951
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Ser Cys Arg Glu Leu Gln Cys Pro Val Gly Val Pro Cys Gln Gln Thr
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ccc cgc ggg ccg cgc tgc gcc tgc ccc cca ggg ttg tcg gga ccc tcc    4047
Pro Arg Gly Pro Arg Cys Ala Cys Pro Pro Gly Leu Ser Gly Pro Ser
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tgc cgc agc ttc ccg ggg tcg ccg ccg ggg gcc agc aac gcc agc tgc    4095
Cys Arg Ser Phe Pro Gly Ser Pro Pro Gly Ala Ser Asn Ala Ser Cys
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gcg gcc gcc ccc tgt ctc cac ggg ggc tcc tgc cgc ccc gcg ccg ctc    4143
Ala Ala Ala Pro Cys Leu His Gly Gly Ser Cys Arg Pro Ala Pro Leu
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gcg ccc ttc ttc cgc tgc gct tgc gcg cag ggc tgg acc ggg ccg cgc    4191
Ala Pro Phe Phe Arg Cys Ala Cys Ala Gln Gly Trp Thr Gly Pro Arg
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tgc gag gcg ccc gcc gcg gca ccc gag gtc tcg gag gag ccg cgg tgc    4239
Cys Glu Ala Pro Ala Ala Ala Pro Glu Val Ser Glu Glu Pro Arg Cys
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ccg cgc gcc gcc tgc cag gcc aag cgc ggg gac cag cgc tgc gac cgc    4287
Pro Arg Ala Ala Cys Gln Ala Lys Arg Gly Asp Gln Arg Cys Asp Arg
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gag tgc aac agc cca ggc tgc ggc tgg gac ggc ggc gac tgc tcg ctg    4335
Glu Cys Asn Ser Pro Gly Cys Gly Trp Asp Gly Gly Asp Cys Ser Leu
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agc gtg ggc gac ccc tgg cgg caa tgc gag gcg ctg cag tgc tgg cgc    4383
Ser Val Gly Asp Pro Trp Arg Gln Cys Glu Ala Leu Gln Cys Trp Arg
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ctc ttc aac aac agc cgc tgc gac ccc gcc tgc agc tcg ccc gcc tgc    4431
Leu Phe Asn Asn Ser Arg Cys Asp Pro Ala Cys Ser Ser Pro Ala Cys
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Leu Tyr Asp Asn Phe Asp Cys His Ala Gly Gly Arg Glu Arg Thr Cys
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Asn Pro Val Tyr Glu Lys Tyr Cys Ala Asp His Phe Ala Asp Gly Arg
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tgc gac cag ggc tgc aac acg gag gag tgc ggc tgg gat ggg ctg gat    4575
Cys Asp Gln Gly Cys Asn Thr Glu Glu Cys Gly Trp Asp Gly Leu Asp
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Cys Ala Ser Glu Val Pro Ala Leu Leu Ala Arg Gly Val Leu Val Leu
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ctg cag cgg ctc agc gcc atc ctg cgc acc tcg ctg cgc ttc cgc ctg    4719
Leu Gln Arg Leu Ser Ala Ile Leu Arg Thr Ser Leu Arg Phe Arg Leu
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gac gcg cac ggc cag gcc atg gtc ttc cct tac cac cgg cct agt cct    4767
Asp Ala His Gly Gln Ala Met Val Phe Pro Tyr His Arg Pro Ser Pro
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Gly Ser Glu Pro Arg Ala Arg Arg Glu Leu Ala Pro Glu Val Ile Gly
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Ser Val Val Met Leu Glu Ile Asp Asn Arg Leu Cys Leu Gln Ser Pro
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Glu Asn Asp His Cys Phe Pro Asp Ala Gln Ser Ala Ala Asp Tyr Leu
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Gly Ala Leu Ser Ala Val Glu Arg Leu Asp Phe Pro Tyr Pro Leu Arg
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Asp Val Arg Gly Glu Pro Leu Glu Pro Pro Glu Pro Ser Val Pro Leu
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ctg cca ctg cta gtg gcg ggc gct gtc ttg ctg ctg gtc att ctc gtc    5055
Leu Pro Leu Leu Val Ala Gly Ala Val Leu Leu Leu Val Ile Leu Val
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ctg ggt gtc atg gtg gcc cgg cgc aag cgc gag cac agc acc ctc tgg    5103
Leu Gly Val Met Val Ala Arg Arg Lys Arg Glu His Ser Thr Leu Trp
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ttc cct gag ggc ttc tca ctg cac aag gac gtg gcc tct ggt cac aag    5151
Phe Pro Glu Gly Phe Ser Leu His Lys Asp Val Ala Ser Gly His Lys
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Gly Arg Arg Glu Pro Val Gly Gln Asp Ala Leu Gly Met Lys Asn Met
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gcc aag ggt gag agc ctg atg ggg gag gtg gcc aca gac tgg atg gac    5247
Ala Lys Gly Glu Ser Leu Met Gly Glu Val Ala Thr Asp Trp Met Asp
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Thr Glu Cys Pro Glu Ala Lys Arg Leu Lys Val Glu Glu Pro Gly Met
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Val Ala Ala Asp Ile Arg Val Ala Pro Ala Met Ala Leu Thr Pro Pro
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Gln Gly Asp Ala Asp Ala Asp Gly Met Asp Val Asn Val Arg Gly Pro
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gat ggc ttc acc ccg cta atg ctg gct tcc ttc tgt ggg ggg gct ctg    5487
Asp Gly Phe Thr Pro Leu Met Leu Ala Ser Phe Cys Gly Gly Ala Leu
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gag cca atg cca act gaa gag gat gag gca gat gac aca tca gct agc    5535
Glu Pro Met Pro Thr Glu Glu Asp Glu Ala Asp Asp Thr Ser Ala Ser
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atc atc tcc gac ctg atc tgc cag ggg gct cag ctt ggg gca cgg act    5583
Ile Ile Ser Asp Leu Ile Cys Gln Gly Ala Gln Leu Gly Ala Arg Thr
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gac cgt act ggc gag act gct ttg cac ctg gct gcc cgt tat gcc cgt    5631
Asp Arg Thr Gly Glu Thr Ala Leu His Leu Ala Ala Arg Tyr Ala Arg
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Ala Asp Ala Ala Lys Arg Leu Leu Asp Ala Gly Ala Asp Thr Asn Ala
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cag gac cac tca ggc cgc act ccc ctg cac aca gct gtc aca gcc gat    5727
Gln Asp His Ser Gly Arg Thr Pro Leu His Thr Ala Val Thr Ala Asp
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gcc cag ggt gtc ttc cag att ctc atc cga aac cgc tct aca gac ttg    5775
Ala Gln Gly Val Phe Gln Ile Leu Ile Arg Asn Arg Ser Thr Asp Leu
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gat gcc cgc atg gca gat ggc tca acg gca ctg atc ctg gcg gcc cgc    5823
Asp Ala Arg Met Ala Asp Gly Ser Thr Ala Leu Ile Leu Ala Ala Arg
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ctg gca gta gag ggc atg gtg gaa gag ctc atc gcc agc cat gct gat    5871
Leu Ala Val Glu Gly Met Val Glu Glu Leu Ile Ala Ser His Ala Asp
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gtc aat gct gtg gat gag ctt ggg aaa tca gcc tta cac tgg gct gcg    5919
Val Asn Ala Val Asp Glu Leu Gly Lys Ser Ala Leu His Trp Ala Ala
            1935                1940                1945

gct gtg aac aac gtg gaa gcc act ttg gcc ctg ctc aaa aat gga gcc    5967
Ala Val Asn Asn Val Glu Ala Thr Leu Ala Leu Leu Lys Asn Gly Ala
        1950                1955                1960
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aat aag gac atg cag gat agc aag gag gag acc ccc cta ttc ctg gcc    6015
Asn Lys Asp Met Gln Asp Ser Lys Glu Glu Thr Pro Leu Phe Leu Ala
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gcc cgc gag ggc agc tat gag gct gcc aag ctg ctg ttg gac cac ttt    6063
Ala Arg Glu Gly Ser Tyr Glu Ala Ala Lys Leu Leu Leu Asp His Phe
1980                1985                1990                1995

gcc aac cgt gag atc acc gac cac ctg gac agg ctg ccg cgg gac gta    6111
Ala Asn Arg Glu Ile Thr Asp His Leu Asp Arg Leu Pro Arg Asp Val
                2000                2005                2010

gcc cag gag aga ctg cac cag gac atc gtg cgc ttg ctg gat caa ccc    6159
Ala Gln Glu Arg Leu His Gln Asp Ile Val Arg Leu Leu Asp Gln Pro
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agt ggg ccc cgc agc ccc ccc ggt ccc cac ggc ctg ggg cct ctg ctc    6207
Ser Gly Pro Arg Ser Pro Pro Gly Pro His Gly Leu Gly Pro Leu Leu
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Cys Pro Pro Gly Ala Phe Leu Pro Gly Leu Lys Ala Ala Gln Ser Gly
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Ser Lys Lys Ser Arg Arg Pro Pro Gly Lys Ala Gly Leu Gly Pro Gln
2060                2065                2070                2075

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Gly Pro Arg Gly Arg Gly Lys Lys Leu Thr Leu Ala Cys Pro Gly Pro
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Leu Ala Asp Ser Ser Val Thr Leu Ser Pro Val Asp Ser Leu Asp Ser
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Pro Arg Pro Phe Gly Gly Pro Pro Ala Ser Pro Gly Gly Phe Pro Leu
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gag ggg ccc tat gca gct gcc act gcc act gca gtg tct ctg gca cag    6495
Glu Gly Pro Tyr Ala Ala Ala Thr Ala Thr Ala Val Ser Leu Ala Gln
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ctt ggt ggc cca ggc cgg gca ggt cta ggg cgc cag ccc cct gga gga    6543
Leu Gly Gly Pro Gly Arg Ala Gly Leu Gly Arg Gln Pro Pro Gly Gly
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tgt gta ctc agc ctg ggc ctg ctg aac cct gtg gct gtg ccc ctc gat    6591
Cys Val Leu Ser Leu Gly Leu Leu Asn Pro Val Ala Val Pro Leu Asp
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tgg gcc cgg ctg ccc cca cct gcc cct cca ggc ccc tcg ttc ctg ctg    6639
Trp Ala Arg Leu Pro Pro Pro Ala Pro Pro Gly Pro Ser Phe Leu Leu
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Pro Gln Glu Arg Pro Pro Pro Tyr Leu Ala Val Pro Gly His Gly Glu
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gag tac ccg gtg gct ggg gca cac agc agc ccc cca aag gcc cgc ttc    6783
Glu Tyr Pro Val Ala Gly Ala His Ser Ser Pro Pro Lys Ala Arg Phe
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Leu Arg Val Pro Ser Glu His Pro Tyr Leu Thr Pro Ser Pro Glu Ser
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Pro Glu His Trp Ala Ser Pro Ser Pro Pro Ser Leu Ser Asp Trp Ser
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Glu Ser Thr Pro Ser Pro Ala Thr Ala Thr Gly Ala Met Ala Thr Thr
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Thr Gly Ala Leu Pro Ala Gln Pro Leu Pro Leu Ser Val Pro Ser Ser
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Lys Arg Gln Val Leu Ala
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