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(54) Title: ANTISENSE OLIGONUCLEOTIDE INHIBITION OF THE ras GENE

(57) Abstract

   Compositions and methods are provided for the modulation of expression of the human ras gene in both the normal and activated forms. Oligonucleotides are provided which are specifically hybridizable with RNA or DNA deriving from the human ras gene, having nucleotide units sufficient in identity and number to effect such specific hybridization. Oligonucleotides specifically hybridizable with human H-ras and human Ki-ras are provided. Such oligonucleotides can be used for diagnostics as well as for research purposes. Methods are also disclosed for modulating ras gene expression in cells and tissues using the oligonucleotides provided, and for specific modulation of expression of the activated ras gene. Methods for diagnosis, detection and treatment of conditions arising from the activation of the H-ras or Ki-ras gene are also disclosed.

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ANTISENSE OLIGONUCLEOTIDE INHIBITION OF THE ras GENE

This application is a continuation-in-part of U.S. Patent
Application No: 715,196, filed June 14, 1991, U.S. Patent
Application No: 958,134, filed October 5, 1992, and U.S. Patent
5   Application No: 08/007,996, filed January 21, 1993, each of
which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to compositions and methods for
the inhibition of expression of the ras gene, a naturally
10 occurring gene which occasionally converts to an activated form
that has been implicated in tumor formation. This invention is
also directed to the specific inhibition of expression of the
activated form of the ras gene. This invention is further
directed to the detection of both normal and activated forms of
15 the ras gene in cells and tissues, and can form the basis for
research reagents and kits both for research and diagnosis.
Furthermore, this invention is directed to treatment of such
conditions as arise from activation of the ras gene. This
invention also relates to stabilized oligonucleotides for
20 inhibition of expression of the ras gene, to such
oligonucleotides which have been further modified to enhance
their affinity for the ras RNA target, and to such
oligonucleotides which have been still further modified to
yield sequence-specific elimination of the ras RNA target.

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BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. There are some
5 thirty families of genes, called oncogenes, which are implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal cell growth
10 and maturation. Mutation of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "activated." It is thought that such a point
15 mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of
20 malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras oncogenes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, K-ras, or N-ras).
25   It is presently believed that inhibiting expression of activated oncogenes in a particular tumor cell might force the cell back into a more normal growth habit. For example, Feramisco et al., *Nature* 1985, 314, 639-642, demonstrated that if cells transformed to a malignant state with an activated ras
30 gene are microinjected with antibody which binds to the protein product of the ras gene, the cells slow their rate of proliferation and adopt a more normal appearance. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of
35 cancer cells.

The H-ras gene has recently been implicated in a serious

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cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is
5 precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a particular variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased
10 risk of sudden cardiac death due to the long Q-T syndrome.

There is a great desire to provide compositions of matter which can modulate the expression of the ras gene, and particularly to provide compositions of matter which specifically modulate the expression of the activated form of
15 the ras gene. It is greatly desired to provide methods of diagnosis and detection of the ras gene in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from ras gene activation. In addition, improved research kits and reagents for detection and study of
20 the ras gene are desired.

Inhibition of oncogene expression has been accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the Ki-ras protooncogene RNA in antisense orientation. Mukhopadhyay, T. et al. (1991) *Cancer Research* 51,
25 1744-1748; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges, R.N. et al. (1993) *Cancer Research*, 53, 1743-1746.

Antisense oligonucleotide inhibition of oncogenes has proven to be a useful tool in understanding the roles of various oncogene families. Antisense oligonucleotides are
30 small oligonucleotides which are complementary to the "sense" or coding strand of a given gene, and as a result are also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt et al., *Mol. Cell Biol.* 1988, 8, 963-973, have shown that antisense
35 oligonucleotides hybridizing specifically with mRNA transcripts of the oncogene c-myc, when added to cultured HL60 leukemic cells, inhibit proliferation and induce differentiation.

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Anfossi et al., *Proc. Natl. Acad. Sci.* 1989, 86, 3379-3383, have shown that antisense oligonucleotides specifically hybridizing with mRNA transcripts of the c-myb oncogene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom
5 et al., *Proc. Nat. Acad. Sci.* 1988, 85, 1028-1032, have shown that expression of the protein product of the c-myc oncogene as well as proliferation of HL60 cultured leukemic cells are inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA. United States Patent No:
10 4,871,838 (Bos et al.) discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect said mutation. United States Patent No: 4,871,838 (Bos et al.) discloses molecules useful as probes for detecting a mutation in DNA which encodes a ras protein.
15   In all these cases, instability of unmodified oligonucleotides has been a major problem, as they are subject to degradation by cellular enzymes. PCT/US88/01024 (Zon et al.) discloses phosphorothioate oligonucleotides hybridizable to the translation initiation region of the amplified c-myc
20 oncogene to inhibit HL-60 leukemia cell growth and DNA synthesis in these cells. Tidd et al., *Anti-Cancer Drug Design* 1988, 3, 117-127, evaluated methylphosphonate antisense oligonucleotides hybridizing specifically to the activated N-ras oncogene and found that while they were resistant to
25 biochemical degradation and were nontoxic in cultured human HT29 cells, they did not inhibit N-ras gene expression and had no effect on these cells. Chang et al. showed that both methylphosphonate and phosphorothioate oligonucleotides hybridizing specifically to mRNA transcripts of the mouse Balb-
30 ras gene could inhibit translation of the protein product of this gene *in vitro*. Chang et al., *Anti-Cancer Drug Design* 1989, 4, 221-232; Brown et al., *Oncogene Research* 1989, 4, 243-252. It was noted that T_m was not well correlated with antisense activity of these oligonucleotides against *in vitro* translation
35 of the ras p21 protein product. Because the antisense oligonucleotides used by Chang et al. hybridize specifically with the translation initiation region of the ras gene, they

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are not expected to show any selectivity for activated ras and the binding ability of these oligonucleotides to normal (wild-type) vs. mutated (activated) ras genes was not compared.

Helene and co-workers have demonstrated selective inhibition of activated (codon 12 G→T transition) H-ras mRNA expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail. This compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations. Saison-Behmoaras, T. et al., *EMBO J.* 1991, 10, 1111-1118. Chang and co-workers disclose selective targeting of mutant H-ras message; this time the target was H-ras codon 61 containing an A→T transversion and the oligonucleotide employed was either an 11-mer methylphosphonate or its psoralen derivative. These compounds, which required concentrations of 7.5-150 μM for activity, were shown by immunoprecipitation to selectively inhibit mutant H-ras p21 expression relative to normal p21. Chang et al., *Biochemistry* 1991, 30, 8283-8286.

Modified nucleotides which increase $\Delta\Delta G°_{37}$ for base mismatches can be used to increase selectivity. It has been found that $\Delta\Delta G°_{37}$ ranges from 1-2 kcal/mol for the most stable mismatches to 5-6 kcal/mol for the least stable mismatches. When possible, therefore, to maximize selectivity for the mutant target, mutations that generate stable mismatches (e.g., G→A) are less preferred than mutations that generate unstable mismatches (e.g., C→G, U→G, A→C). An example of this can be found in the autosomal dominant mutations associated with familial Alzheimer's disease. Three different point mutations of the β-amyloid precursor gene have been shown to cosegregate with this disease. These mutations include G→A ($\Delta\Delta G°_{37}$ = +1.2 kcal/mol), G→T ($\Delta\Delta G°_{37}$ = +3.9 kcal/mol), and T→G ($\Delta\Delta G°_{37}$ = +6.3 kcal/mol)[2]. Goate et al., *Nature* 1991, 349, 704-706; Murrel et al., *Science* 1991, 254, 97-99; Chartier-Harlin et al., *Nature* 1991, 353, 844-846. In this case, targeting the T→G mutation is believed to yield the greatest selectivity for mutant β-amyloid by an antisense oligonucleotide.

DNA oligonucleotides having unmodified phosphodiester

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internucleoside linkages or modified phosphorothioate internucleoside linkages are substrates for cellular RNase H; i.e., they activate the cleavage of target RNA by the RNase H. (Dagle, J.M, Walder, J.A. and Weeks, D.L., *Nucleic Acids*

5 *Research* **1990**, *18*, 4751; Dagle, J.M., Weeks, D.L. and Walder, J.A., *Antisense Research And Development* **1991**, *1*, 11; and Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A., *Nucleic Acids Research* **1991**, *19*, 1805). RNase H is an endonuclease that cleaves the RNA strand of RNA:DNA duplexes;

10 activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the ability of antisense oligonucleotides to inhibit target RNA expression. Walder et al. note that in *Xenopus* embryos, both phosphodiester linkages and phosphorothioate linkages are also subject to exo-

15 nuclease degradation. Such nuclease degradation is detrimental since it rapidly depletes the oligonucleotide available for RNase H activation. PCT Publication WO 89/05358, Walder et al., discloses DNA oligonucleotides modified at the 3' terminal internucleoside linkage to make them resistant to nucleases

20 while remaining substrates for RNAse H.

Attempts to take advantage of the beneficial properties of oligonucleotide modifications while maintaining the substrate requirements for RNase H have led to the employment of chimeric oligonucleotides. Giles, R.V. et al., *Anti-Cancer*

25 *Drug Design* **1992**, *7*, 37; Hayase, Y. et al., *Biochemistry* **1990**, *29*, 8793; Dagle, J.M. et al., *Nucleic Acids Research* **1990**, *18*, 4751; Dagle, J.M. et al., *Nucleic Acids Research* **1991**, *19*, 1805. Chimeric oligonucleotides contain two or more chemically distinct regions, each comprising at least one nucleotide.

30 These oligonucleotides typically contain a region of modified nucleotides that confer one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and an unmodified region that retains the ability to

35 direct RNase H cleavage. This approach has been employed for a variety of backbone modifications, most commonly methylphosphonates, which alone are not substrates for RNAse H.

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Methylphosphonate oligonucleotides containing RNase H-sensitive phosphodiester linkages were found to be able to direct target RNA cleavage by RNase H *in vitro*. Using *E. coli* RNase H, the minimum phosphodiester length required to direct efficient

5 RNase H cleavage of target RNA strands has been reported to be either three or four linkages. Quartin, R.S. et al. *Nucleic Acids Research* **1989**, 17, 7253; Furdon, P.J. et al. *Nucleic Acids Research* **1989**, 17, 9193. Similar studies have been reported using *in vitro* mammalian RNase H cleavage assays.

10 Agrawal, S. et al., *Proc. Natl. Acad. Sci. USA* **1990**, 87, 1401. In this case, a series of backbone modifications, including methylphosphonates, containing different phosphodiester lengths were examined for cleavage efficiency. The minimum phosphodiester length required for efficient RNase H cleavage

15 directed by oligonucleotides of this nature is five linkages. More recently, it has been shown that methylphosphonate/ phosphodiester chimeras display increased specificity and efficiency for target RNA cleavage using *E. coli* RNase H *in vitro*. Giles, R.V. et al., *Anti-Cancer Drug Design* **1992**, 7,

20 37. These compounds have also been reported to be effective antisense inhibitors in *Xenopus* oocytes and in cultured mammalian cells. Dagle, J.M. et al., *Nucleic Acids Res.* **1990**, 18, 4751; Potts, J.D., et al., *Proc. Natl. Acad. Sci. USA* **1991**, 88, 1516.

25   PCT Publication WO 90/15065, Froehler et al., discloses chimeric oligonucleotides "capped" at the 3' and/or the 5' end by phosphoramidite, phosphorothioate or phosphorodithioate linkages in order to provide stability against exonucleases while permitting RNAse H activation. PCT Publication WO

30 91/12323, Pederson et al., discloses chimeric oligonucleotides in which two regions with modified backbones (methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates or phosphoramidates) which do not activate RNAse H flank a central deoxynucleotide region which does activate RNAse H cleavage.

35 2'-deoxy oligonucleotides have been stabilized against nuclease degradation while still providing for RNase H activation by positioning a short section of phosphodiester linked

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nucleotides between sections of backbone-modified oligonucleotides having phosphoramidate, alkylphosphonate or phosphotriester linkages. Dagle, J.M, Walder, J.A. and Weeks, D.L., *Nucleic Acids Research* 1990, 18, 4751; Dagle, J.M.,
5 Weeks, D.L. and Walder, J.A., *Antisense Research And Development* 1991, 1, 11; and Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A., *Nucleic Acids Research* 1991, 19, 1805. While the phosphoramidate containing oligonucleotides were stabilized against exonucleases, each phosphoramidate
10 linkage resulted in a loss of 1.6°C in the measured T_m value of the phosphoramidate containing oligonucleotides. Dagle, J.M., Andracki, M.E., DeVine, R.J. and Walder, J.A., *Nucleic Acids Research* 1991, 19, 1805. Such loss of the T_m value is indicative of a decrease in the hybridization between the
15 oligonucleotide and its target strand.

Saison-Behmoaras, T., Tocque, B. Rey, I., Chassignol, M., Thuong, N.T. and Helene, C., *EMBO Journal* 1991, 10, 1111, observed that even though an oligonucleotide was a substrate for RNase H, cleavage efficiency by RNase H was low because of
20 weak hybridization to the mRNA.

Chimeric oligonucleotides are not limited to backbone modifications, though chimeric oligonucleotides containing 2' ribose modifications mixed with RNase H-sensitive deoxy residues have not been as well characterized as the backbone
25 chimeras. EP Publication 260,032 (Inoue et al.) and Ohtsuka et al., *FEBS Lett.* 1987, 215, 327-330, employed 2'-O-methyl oligonucleotides (which alone would not be substrates for RNAse H) containing unmodified deoxy gaps to direct cleavage *in vitro* by *E. coli* RNase H to specific sites within the complementary
30 RNA strand. These compounds required a minimum deoxy gap of four bases for efficient target RNA cleavage. However, oligonucleotides of this nature were not examined for cleavage efficiency using mammalian RNase H nor tested for antisense activity in cells. These oligonucleotides were not stabilized
35 against nucleases.

Studies on the ability to direct RNase H cleavage and antisense activity of 2' ribose modifications other than O-

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methyl have been extremely limited. Schmidt, S. et al.,
Biochim. Biophys. Acta **1992**, 1130, 41.

While it has been recognized that cleavage of a target
RNA strand using an antisense oligonucleotide and RNase H would
5 be useful, nuclease resistance of the oligonucleotide and
fidelity of the hybridization are also of great importance.
There has been a long-felt need for methods or materials that
could both activate RNase H while concurrently maintaining or
improving hybridization properties and providing nuclease
10 resistance. There remains a long-felt need for such methods
and materials for enhancing antisense activity.

OBJECTS OF THE INVENTION

It is an object of the invention to provide
oligonucleotides complementary to ras mRNA which inhibit
15 expression of the ras gene.

It is another object of the invention to provide
oligonucleotides complementary to ras mRNA which specifically
inhibit expression of an activated (mutant) form of the ras
gene.
20 Yet another object of the invention is to provide
stabilized oligonucleotides which inhibit expression of the ras
gene.

Another object of the invention is to provide stabilized
oligonucleotides complementary to ras mRNA and modified to
25 increase their affinity for the ras mRNA target, which inhibit
expression of the ras gene.

Still another object is to provide oligonucleotides which
are complementary to ras mRNA and which are substrates for
RNAse H.
30 An additional object of the invention is to provide
oligonucleotides which inhibit proliferation of cancer cells.
Methods of inhibiting proliferation of cancer cells are also an
object of this invention.

Detection of the mutation from the normal (wild-type) to
35 activated form of the ras gene is another object of the
invention.

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Differential diagnosis of morphologically similar tumors and identification of high-risk conditions based on the presence of the activated ras gene is yet another object of this invention.

5   A further object of this invention is to provide methods of diagnosis and treatment of conditions arising due to mutation of the gene from the wild-type to the mutant, activated form of the ras gene.

SUMMARY OF THE INVENTION

10   In accordance with the present invention, oligonucleotides are provided that are complementary to DNA or RNA deriving from the human ras gene. In one preferred embodiment, oligonucleotides that are complementary to DNA or RNA deriving from the human H-ras gene are provided. It is

15 preferred that these oligonucleotides be complementary to the translation initiation codon of the gene, and preferably that the oligonucleotides comprise a sequence CAT. In accordance with another preferred embodiment, oligonucleotides that are complementary to codon 12 of the activated H-ras gene are

20 provided, preferably comprising a sequence GAC. In another such embodiment, oligonucleotides are provided that are complementary to and hybridize preferentially with the mutated codon 12 of the activated H-ras gene. In this embodiment, such oligonucleotide preferably comprises a sequence GAC. Such

25 oligonucleotides are conveniently and desirably presented in a pharmaceutically acceptable carrier. In another preferred embodiment, oligonucleotides that are complementary to DNA or RNA deriving from the human Ki-ras gene are provided. It is preferred that these oligonucleotides be complementary to the

30 5'-untranslated region, 3'-untranslated region, codon 12 or codon 61 of the Ki-ras gene. In accordance with another preferred embodiment, oligonucleotides that are complementary to codon 12 of the activated Ki-ras gene are provided, preferably comprising a sequence ACC. In another such

35 embodiment, oligonucleotides are provided that are complementary to and hybridize preferentially with the mutated

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codon 12 of the activated Ki-ras gene. In this embodiment, such oligonucleotide preferably comprises a sequence AAC. Such oligonucleotides are conveniently and desirably presented in a pharmaceutically acceptable carrier.

5   It is preferred that the oligonucleotides are modified to increase their resistance to degradation by nucleases. It is presently preferred that increased resistance to nucleases is conveyed by at least one sulfur-containing nucleotide, most preferably a phosphorothioate or phosphorodithioate.

10   In accordance with other preferred embodiments, oligonucleotides complementary to ras mRNA are provided which inhibit ras expression and which, at once, have increased resistance to nucleases, have increased binding affinity for the ras mRNA target, and are substrates for RNAse H.

15   It is presently preferred that increased binding affinity is conveyed by modification of at least one nucleotide at the 2' position of the sugar, most preferably comprising a 2'-O-alkyl, 2'-O-alkylamino or 2'-fluoro modification.

          In some preferred embodiments, the oligonucleotides of 20 the invention are chimeric oligonucleotides comprising at least one region which is modified to increase binding affinity for the complementary ras mRNA, and a region which is a substrate for RNAse H cleavage. In one such embodiment an RNAse H substrate region is flanked by two regions having increased ras 25 mRNA binding affinity.

          Other aspects of the invention are directed to methods for modulating the expression of the human ras gene in cells or tissues and for specifically modulating the expression of the activated ras gene in cells or tissues suspected of harboring 30 a mutation leading to such activation.

          Some embodiments of the invention are directed to methods for inhibiting the expression of the ras gene and for specifically inhibiting the expression of the activated ras gene.

35   Additional aspects of the invention are directed to methods of detection of the ras gene in cells or tissues and specific detection of the activated ras gene in cells or

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tissues suspected of harboring said mutated gene. Such methods comprise contacting cells or tissues suspected of containing the human ras gene with oligonucleotides in accordance with the invention in order to detect said gene.

5   Other aspects of the invention are directed to methods for diagnostics and therapeutics of animals suspected of having a mutation leading to activation of the ras gene. Such methods comprise contacting the animal or cells or tissues or a bodily fluid from the animal with oligonucleotides in accordance with 10 the invention in order to inhibit the expression of this gene, to treat conditions arising from activation of this gene, or to effect a diagnosis thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing dose-response inhibition 15 of ras-luciferase fusion protein expression using oligonucleotides targeted to the H-ras translation initiation codon (AUG). Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when luciferin is added.

20   Figure 2 is a bar graph showing dose-response inhibition of ras-luciferase fusion protein expression using oligonucleotides targeted to the mutated codon-12 region in activated H-ras. Expression is measured by measurement of luciferase activity as assayed by amount of light emitted when 25 luciferin is added.

Figure 3 is a bar graph showing single-dose inhibition of ras-luciferase fusion protein expression by antisense phosphorothioate compounds. Expression is measured by measurement of luciferase activity as assayed by amount of 30 light emitted when luciferin is added.

Figure 4 is a table and bar graph summarizing data obtained for 13 antisense oligonucleotides specifically hybridizable with the activated H-ras gene. Shown for each oligonucleotide is its length, region of the activated ras gene 35 to which it specifically hybridizes, and its activity in inhibiting expression of the ras-luciferase fusion protein.

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Figure 5 shows the ras mRNA target sequence (shown 5' to 3') and locations and sequences of antisense oligonucleotides targeted to the H-ras translation initiation codon (AUG) and the codon 12 region. Antisense oligonucleotides are shown 3' to 5'. Figure 5A shows two 20-mers (2502 and 2503) targeted to the AUG and a series of oligonucleotides from 5 to 25 nucleotides in length, targeted to codon 12. Figure 5B shows oligonucleotides 2502, 2503, 6186 and 2570 in relation to the ras mRNA target sequence.

Figure 6 is a bar graph showing inhibition of ras-luciferase by various doses of oligonucleotides 2502, 2503, 6186 and uniformly 2'-O-methylated versions of these phosphorothioate oligonucleotides.

Figure 7 is a bar graph showing antisense inhibition of mutant (striped bars) and normal (solid bars) ras-luciferase by antisense oligonucleotides of various lengths.

Figure 8 is a series of 8 panels showing inhibition of ras in a dose-dependent manner. Solid lines are activity against wild-type, dotted lines show activity against activated ras.

Figure 9 is a two-part figure showing antisense oligonucleotide binding to the 47-mer H-ras RNA hairpin target. Figure 9A is a gel shift analysis of hairpin target with uniform 2'-O-methyl oligonucleotide (deoxy number = 0) and of hairpin target with a 2'-O-methyl chimeric oligonucleotide having a nine base deoxy gap (deoxy number = 9) as a function of oligonucleotide concentration. Lanes 1-8 contain the following oligonucleotide concentrations: 1) none; 2) 10^{-11}M; 3) 10^{-10}M; 4) 10^{-9}M; 5) 10^{-8}M; 6) 10^{-7}M; 7) 10^{-6}M; 8) 10^{-5}M. Figure 9B is a graph showing fraction of hairpin target shifted vs. concentration of antisense oligonucleotide. ◊: Deoxy number= 17; •: Deoxy number= 9; ▲: Deoxy number= 7; O: Deoxy number= 5; △: Deoxy number= 3; ■: Deoxy number= 1; □: Deoxy number= 0. (Inset: structure of 47-mer H-ras hairpin target shown with sequence of oligonucleotide 2570).

Figure 10 is a gel showing RNAse H dependent cleavage of complementary   H-ras   RNA   by   2'-O-methyl   chimeric

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phosphorothioate oligonucleotides. Lane designations refer to the length of the centered deoxy gap.

Figure 11 is a two-part figure showing antisense activity of phosphorothioate 2'-O-methyl chimeric oligonucleotides targeted to ras codon-12 RNA sequences. Figure 11A is a bar graph showing single-dose activity (100 nM) of uniform 2'-O-methyl oligonucleotides, uniform deoxy oligonucleotides and chimeric 2'-O-methyl oligonucleotides containing centered 1-, 3-, 5-, 7- or 9-base deoxy gaps. Figure 11B is a line graph showing dose-response activity of uniform deoxy (▼) or 2'-O-methyl oligonucleotides containing centered 4-(■,♦), 5-(•), 7-(+) or 9-base (▲) deoxy gaps.

Figure 12 is a bar graph showing antisense activities of a uniform deoxy phosphorothioate and shortened chimeric oligonucleotides against ras-luciferase.

Figure 13 is a line graph showing correlation between antisense activity and ability to activate RNAse H as a function of deoxy gap length using phosphorothioate 2'-O-methyl oligonucleotides targeted against ras.

Figure 14 is a line graph showing does response antisense activities of phosphorothioate 2'-modified chimeric oligonucleotides containing 7-base deoxy gaps. (▲), uniform deoxy phosphorothioate; (■), 2'-O-pentyl chimera; (•), 2'-O-propyl chimera; (♦), 2'-O-methyl chimera; (▼), 2'-fluoro chimera.

Figure 15 is a bar graph showing dose-dependent oligonucleotide inhibition of ras-luciferase by chimeric oligonucleotides having various combinations of phosphorothioate and phosphodiester backbones and 2'-O-methyl and 2'-deoxy nucleotides.

Figure 16 is a line graph showing anti-tumor activity of ISIS 2503 against A549 human cell tumors in nude mice.

Figure 17 is a line graph showing anti-tumor activity of ras oligo ISIS 2503, administered with cationic lipid, against A549 human cell tumors in nude mice.

Figure 18 is a bar graph showing activity against Ha-ras of oligonucleotides with various 2' sugar modifications and

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phosphodiester   (P=O)   backbones   compared   to   a
2'deoxyoligonucleotide with phosphorothioate (P=S) backbone.

        Figure 19 is a bar graph showing antisense inhibition of
Ki-ras mRNA expression in three human colon carcinoma cell
5   lines, Calu1, SW480 and SW620.

        Figure 20 is a bar graph showing inhibition of SW480
human carcinoma cell line proliferation by Ki-ras specific
oligonucleotides ISIS 6957 and ISIS 6958.

        Figure 21 is a bar graph showing selective inhibition of
10   Ki-ras mRNA expression in human carcinoma SW480 cells, which
express mutant Ki-ras, compared to HeLa cells, which express
wild-type Ki-ras, when treated with oligonucleotide targeted to
the mutant Ki-ras codon-12.

DETAILED DESCRIPTION OF THE INVENTION

15   Malignant tumors develop through a series of stepwise,
progressive changes that lead to the loss of growth control
characteristic of cancer cells, i.e., continuous unregulated
proliferation, the ability to invade surrounding tissues, and
the ability to metastasize to different organ sites. Carefully
20   controlled *in vitro* studies have helped define the factors that
characterize the growth of normal and neoplastic cells and have
led to the identification of specific proteins that control
cell growth and differentiation. In addition, the ability to
study cell transformation in carefully controlled, quantitative
25   *in vitro* assays has led to the identification of specific genes
capable of inducing the transformed cell phenotype.   Such
cancer-causing genes, or oncogenes, are believed to acquire
transformation-inducing properties through mutations leading to
changes in the regulation of expression of their protein
30   products.   In some cases such changes occur in non-coding DNA
regulatory domains, such as promoters and enhancers, leading to
alterations in the transcriptional activity of oncogenes,
resulting in over- or under-expression of their gene products.
In other cases, gene mutations occur within the coding regions
35   of oncogenes, leading to the production of altered gene
products that are inactive, overactive, or exhibit an activity

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that is different from the normal (wild-type) gene product.

To date, more than 30 cellular oncogene families have been identified. These genes can be categorized on the basis of both their subcellular location and the putative mechanism of action of their protein products. The ras oncogenes are members of a gene family which encode related proteins that are localized to the inner face of the plasma membrane. ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The sequences of H-ras, K-ras and N-ras are known. Capon et al., *Nature 302* 1983, 33-37; Kahn et al., *Anticancer Res.* 1987, 7, 639-652; Hall and Brown, *Nucleic Acids Res.* 1985, 13, 5255-5268. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. Tabin, C.J. et al., *Nature* 1982, 300, 143-149; Reddy, P.E. et al., *Nature* 1982, 300, 149-152; Taparowsky, E. et al., *Nature* 1982, 300, 762-765. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The present invention provides oligonucleotides for inhibition of human ras gene expression. Such oligonucleotides specifically hybridize with selected DNA or mRNA deriving from a human ras gene. The invention also provides oligonucleotides

5 for selective inhibition of expression of the mutant form of ras.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes

10 oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example,

15 enhanced cellular uptake and increased stability in the presence of nucleases.

Specific examples of some preferred oligonucleotides envisioned for this invention may contain phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or

20 cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with $CH_2-NH-O-CH_2$, $CH_2-N(CH_3)-O-CH_2$, $CH_2-O-N(CH_3)-CH_2$, $CH_2-N(CH_3)-N(CH_3)-CH_2$ and $O-N(CH_3)-CH_2-CH_2$ backbones (where phosphodiester is $O-P-O-CH_2$). Also preferred are oligonucleotides having

25 morpholino backbone structures. Summerton, J.E. and Weller, D.D., U.S. Patent No: 5,034,506. In other preferred embodiments, such as the protein-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound

30 directly or indirectly to the aza nitrogen atoms of the polyamide backbone. P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt, *Science* 1991, 254, 1497. Other preferred oligonucleotides may contain alkyl and halogen-substituted sugar moieties comprising one of the following at the 2'

35 position: OH, SH, SCH_3, F, OCN, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-

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alkyl; O-, S-, or N-alkenyl; SOCH$_3$; SO$_2$CH$_3$; ONO$_2$; NO$_2$; N$_3$; NH$_2$;
heterocycloalkyl; heterocycloalkaryl; aminoalkylamino;
polyalkylamino; substituted silyl; an RNA cleaving group; a
conjugate; a reporter group; an intercalator; a group for
5 improving the pharmacokinetic properties of an oligonucleotide;
or a group for improving the pharmacodynamic properties of an
oligonucleotide and other substituents having similar
properties. Oligonucleotides may also have sugar mimetics such
as cyclobutyls in place of the pentofuranosyl group.
10   Other preferred embodiments may include at least one
modified base form. Some specific examples of such modified
bases include 2-(amino)adenine, 2-(methylamino)adenine, 2-
(imidazolylalkyl)adenine, 2-(aminoalklyamino)adenine or other
heterosubstituted alkyladenines.
15   Preferred oligonucleotides of this invention may, at
once, comprise nucleotides modified to increase their
resistance to nucleases, comprise nucleotides modified to
increase their affinity for ras mRNA, and comprise nucleotides
which are substrates for RNAse H. In one preferred embodiment,
20 a chimeric oligonucleotide comprises at least one region
modified to increase ras mRNA binding affinity, and a region
which is a substrate for RNAse H. The oligonucleotide is also
modified to enhance nuclease resistance. In a more preferred
embodiment, the region which is a substrate for RNAse H is
25 flanked by two regions which are modified to increase ras mRNA
binding affinity. The effect of such modifications is to
greatly enhance antisense oligonucleotide inhibition of ras
gene expression.
        The oligonucleotides in accordance with this invention
30 preferably comprise from about 8 to about 50 nucleic acid base
units. It is more preferred that such oligonucleotides
comprise from about 8 to 30 nucleic acid base units, and still
more preferred to have from about 13 to 25 nucleic acid base
units. As will be appreciated, a nucleic acid base unit is a
35 base-sugar combination suitably bound to adjacent nucleic acid
base unit through phosphodiester or other bonds.
        "Hybridization," in the context of this invention, means

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hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to
5 form three hydrogen bonds between them.

"Specifically hybridizable" indicates a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences. It is understood that an oligonucleotide need not be 100% complementary to its target
10 nucleic acid sequence to be specifically hybridizable.

Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression: A series of antisense phosphorothioate oligonucleotides targeted to either the H-ras translation initiation codon or the codon-12 point mutation of activated H-
15 ras were screened using the ras-luciferase reporter gene system described in Examples 2-5. Of this initial series, six oligonucleotides were identified that gave significant and reproducible inhibition of ras-luciferase activity. The base sequences, sequence reference numbers and SEQ ID numbers of
20 these oligonucleotides (all are phosphorothioates) are shown in Table 1.

TABLE 1

OLIGO REF NO	SEQUENCE	SEQ ID NO:
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

Figure 1 shows a dose-response experiment in which cells expressing either the normal ras-luciferase reporter gene or the mutant ras-luciferase reporter gene were treated with increasing concentrations of the phosphorothioate
35 oligonucleotide 2503 (SEQ ID NO: 2). This compound is targeted

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to the translational initiation codon of H-ras RNA transcripts. As shown in Figure 1, treatment of cells with this oligonucleotide resulted in a dose-dependent inhibition of ras-luciferase activity, displaying IC50 values of approximately 50

5 nM for both the normal and the mutant ras targets. The control oligonucleotide is a random phosphorothioate oligonucleotide, 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. The observation that an oligonucleotide

10 targeted to the ras translation initiation codon is equally effective in reducing both mutant and normal ras expression is expected since the two targets have identical sequence compositions in the region surrounding the AUG translation initiation site.

15   Figure 2 shows a dose-response experiment in which cells were treated with phosphorothioate oligonucleotide 2570 (SEQ ID NO: 3), a compound that is targeted to the codon-12 point mutation of mutant (activated) H-ras RNA. The control oligonucleotide is a random phosphorothioate oligonucleotide,

20 20 bases long. Results are expressed as percentage of luciferase activity in transfected cells not treated with oligonucleotide. As the figure shows, treatment of cells with increasing concentrations of this oligonucleotide resulted in a dose-dependent inhibition of ras-luciferase activity in cells

25 expressing either the mutant form or the normal form of ras-luciferase. However, careful examination of the data shows that at low concentrations, oligonucleotide 2570 displayed approximately threefold selectivity toward the mutant form of ras-luciferase as compared to the normal form. In fact, 2570

30 displayed an IC50 value for the mutant form of ras-luciferase of approximately 100 nM whereas the same compound displayed in IC50 value of approximately 250 nM for the unmutated form.

       Figure 3 shows the results of a typical experiment in which cells expressing either the normal form or the mutant

35 form of ras-luciferase were treated with a single dose (0.5 μM) of oligonucleotide targeted to either the translation initiation codon of H-ras or the codon-12 point mutation. The

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antisense phosphorothioate oligonucleotides tested are shown in
Table 1. The control oligonucleotide (2504) is a random
phosphorothioate oligonucleotide, 20 bases long. Results are
expressed as percentage of luciferase activity in transfected
5   cells not treated with oligonucleotide. As shown in Figure 3,
compound 2503 (SEQ ID NO: 2), targeted to the ras translational
initiation codon, was most effective in inhibiting ras-
luciferase activity. Of the three compounds targeted to the
codon-12 point mutation of activated H-ras, only the 17-mer
10   oligonucleotide 2570 (SEQ ID NO: 3) displayed selectivity
toward the mutated form of ras-luciferase as compared to the
normal form. This is also shown in Figure 4, which summarizes
data obtained with all 13 antisense oligonucleotides
complementary to the activated H-ras gene, as well as a
15   scrambled control oligonucleotide (1966) and a control
oligonucleotide (2907) complementary to the codon-12 region of
wild-type ras. Shown for each oligonucleotide is its length,
region to which it is complementary, and its activity in
suppressing expression of the ras-luciferase fusion protein.
20   The longer phosphorothioates targeted to the codon-12 point
mutation, while displaying substantial antisense activity
toward ras-luciferase expression, did not demonstrate selective
inhibition of expression of the mutant form of ras-luciferase.
Phosphorothioate oligonucleotides targeted to the codon-12
25   point mutation that were less than 17 nucleotides in length did
not show activity to either form of ras-luciferase. These
results demonstrate effective antisense activity of
phosphorothioate oligonucleotides targeted to ras sequences.

     Antisense oligonucleotides specifically hybridizable with
30   **the H-ras AUG:** Three 20-base phosphorothioate oligonucleotides,
targeted to the H-ras AUG codon, were compared for their
ability to inhibit ras-luciferase expression in transient
transfection assays as described in Examples 2-5. Results are
shown in Figures 5A and 5B. These oligonucleotides, ISIS 2502
35   (SEQ ID NO: 1), 2503 (SEQ ID NO: 2) and 6186 (SEQ ID NO: 7)
shown in Table 2, were tested for inhibition of ras-luciferase
expression at a single dose (100 nM) in HeLa cells. All three

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AUG-targeted oligonucleotides were effective in inhibiting ras-luciferase expression. These three phosphorothioate oligonucleotides were also prepared with a 2'-O-methyl modification on each sugar. The 2'-O-methylated version of
5 ISIS 2503 (SEQ ID NO: 2) also inhibited ras-luciferase expression. This is shown in Figure 6.

TABLE 2

Antisense oligonucleotides targeted to mutant H-ras
(Oligonucleotide sequences shown 5' to 3')

OLIGO	LENGTH	TARGET	SEQUENCE	SEQ. ID NO.
2502	20	AUG	CTTATATTCCGTCATCGCTC	1
2503	20	AUG	TCCGTCATCGCTCCTCAGGG	2
6186	20	AUG	TATTCCGTCATCGCTCCTCA	7
2563	5	CODON 12	CGACG	8
2564	7	CODON 12	CCGACGG	9
2565	9	CODON 12	ACCGACGGC	10
2567	11	CODON 12	CACCGACGGCG	11
2568	13	CODON 12	ACACCGACGGCGC	12
2569	15	CODON 12	CACACCGACGGCGCC	13
3426	16	CODON 12	CCACACCGACGGCGCC	14
3427	16	CODON 12	CACACCGACGGCGCCC	15
2570	17	CODON 12	CCACACCGACGGCGCCC	3
3428	18	CODON 12	CCCACACCGACGGCGCCC	16
3429	18	CODON 12	CCACACCGACGGCGCCCA	17
2571	19	CODON 12	CCCACACCGACGGCGCCCA	4
2566	21	CODON 12	GCCCACACCGACGGCGCCCAC	5
2560	23	CODON 12	TGCCCACACCGACGGCGCCCACC	6
2561	25	CODON 12	TTGCCCACACCGACGGCGCCCACCA	18
907	17	CODON 12 (wild type)	CCACACCGCCGGCGCCC	19

Oligonucleotide length affects antisense activity and specificity: Oligonucleotides targeted to the H-ras codon-12 point mutation also were effective in inhibiting expression of ras-luciferase. A series of eleven phosphorothioate
35 oligonucleotides, ranging in length between 5 and 25 bases, were made and tested for ability to inhibit mutant and wild

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type ras-luciferase in transient transfection assays as described in Examples 2-5. The oligonucleotides are shown in Table 2. At 100 nM oligonucleotide concentration, oligonucleotides 15 bases or greater in length were found to
5 inhibit expression of the mutant H-ras target. Selective inhibition of mutant over wild type ras-luciferase expression was observed for oligonucleotides between 15 and 19 bases in length. The maximum selectivity observed for inhibition of mutant ras-luciferase relative to wild type was for the 17-mer
10 2570 (SEQ ID NO: 3) and was approximately 4-fold. In order to demonstrate that 2570 was acting in a sequence-specific manner, a variant of this compound was tested (2907; SEQ ID NO: 19) in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the wild
15 type H-ras target. Hence, this oligonucleotide will contain a single mismatch at the center of the oligonucleotide/RNA duplex when fully hybridized to the mutant H-ras sequence. As shown in Figure 7, oligonucleotide 2907 selectively inhibited expression of wild type ras-luciferase relative to mutant ras-
20 luciferase, with the difference being approximately 5-fold at an oligonucleotide dosage of 100 nM.

Two 16-mers and two 18-mers complementary to the mutant codon-12 region (Figure 5 and Table 2) were tested as described in Examples 2-5. Figure 8 shows the results of an experiment
25 in which antisense activity and mutant selectivity was determined for oligonucleotides of length 13, 15, 16, 17, 18 and 19 bases in a dose-dependent manner. The results obtained with these oligonucleotides demonstrated that the compounds that were active against mutant H-ras sequences also showed
30 selectivity; oligonucleotides of length 16 (SEQ ID NO: 14 and SEQ ID NO: 15) and 17 bases (SEQ ID NO: 3) displayed the greatest selectivity (4- and 5-fold, respectively). The 13 base compound, 2568 (SEQ ID NO: 12), did not display antisense activity at any of the tested concentrations.
35   **Chimeric 2'-O-methyl oligonucleotides with deoxy gaps:** Based on the sequence of the mutant-selective 17-mer (2570), a series of chimeric phosphorothioate 2'-O-methyl

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oligonucleotides were synthesized in which the end regions consisted of 2'-O-methyl nucleosides and the central residues formed a "deoxy gap". The number of deoxy residues ranged from zero (full 2'-O-methyl) to 17 (full deoxy). These oligonucleotides are shown in Table 3.

TABLE 3

Chimeric phosphorothioate oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (Mutant codon-12 target)

OLIGO #	DEOXY	SEQUENCE	SEQ ID NO
4122	0	CCACACCGACGGCGCCC	3
3975	1	CCACACCGACGGCGCCC	3
3979	3	CCACACCGACGGCGCCC	3
4236	4	CCACACCGACGGCGCCC	3
4242	4	CCACACCGACGGCGCCC	3
3980	5	CCACACCGACGGCGCCC	3
3985	7	CCACACCGACGGCGCCC	3
3984	9	CCACACCGACGGCGCCC	3
2570	17	CCACACCGACGGCGCCC	3

These oligonucleotides were characterized for hybridization efficiency as described in Example 6, ability to direct RNase H cleavage *in vitro* using mammalian RNase H as described in Example 8, and for antisense activity. Antisense activity against full length H-ras mRNA was determined using a transient co-transfection reporter gene system in which H-ras gene expression was monitored using a ras-responsive enhancer element linked to the reporter gene luciferase, as described in Example 9.

Hybridization of phosphorothioate antisense oligonucleotides to single stranded 25-mer RNA targets: Figure 5 and Table 2 show the sequences of 15 phosphorothioate oligonucleotides targeted to activated H-ras mRNA containing the codon 12 G→U point mutation. These oligonucleotides range between 5 and 25 bases in length and are centered around the point mutation. Melting temperatures (T_m) for these antisense

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phosphorothioates against mutant and wild type 25-mer RNA targets at 4 μM oligonucleotide concentration were measured. T_m increased with increasing chain length and, for any chain length, T_m for hybridization to the mutant target was greater

5 than that for the wild type target. Oligonucleotide 2907 is a phosphorothioate 17-mer variant of 2570 in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the wild type H-ras target. As expected, the melting temperature for hybridization

10 of this oligonucleotide to the wild type target was greater than that for the mutant target, which now contains a single mismatch in the oligonucleotide/RNA duplex at the site of the point mutation. For the 17-mer phosphorothioate (2570) that is perfectly complementary to the mutant H-ras target,

15 thermodynamic parameters were also obtained from dependence of T_m on oligonucleotide concentration. These data were used to determine the free energy difference ($\Delta\Delta G°_{37}$) between hybridization of oligonucleotides to the mutant target and to the wild type target. For a given oligonucleotide, $\Delta\Delta G°_{37}$ can

20 be obtained from T_m dependence on oligonucleotide concentration. Borer, P.N. et al., *J. Mol. Biol.* 1974, 86, 843-853. The $\Delta\Delta G°_{37}$ for 2570 was calculated to be +1.8 kcal/mole.

The maximum degree of selectivity that can be achieved

25 for targeting mutant over wild type ras increases significantly as $\Delta\Delta G°_{37}$ increases. Therefore, chemical modifications of the antisense oligonucleotide which increase $\Delta\Delta G°_{37}$ enhance selectivity. One such modification is 2,6-diaminopurine, which is believed to bind more tightly than dA to U and less tightly

30 than dA to G, and thus to increase $\Delta\Delta G°_{37}$ for the A-U -->A-G mismatch. The substrate requirements of RNase H can also be exploited to obtain selectivity according to the teachings of this invention. If the enzyme is unable to bind or cleave a mismatch, additional selectivity will be obtained beyond that

35 conferred by $\Delta\Delta G°_{37}$ by employing chimeric oligonucleotides that place the RNAse H recognition site at the mismatch. This has been found to be the case; RNase H can indeed discriminate

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between a fully matched duplex and one containing a single mismatch.

      Hybridization of "deoxy gap" oligonucleotides to short oligonucleotide targets: Hybridization analysis of the 2'-O-
5 methyl deoxy gap series against a 25-mer synthetic oligoribonucleotide complement as described in Example 6 demonstrated that T_m values for a given oligonucleotide correlated directly with 2'-O-methyl content. As 2'-O-methyl modifications were replaced with deoxy substituents, T_m values
10 were reduced at approximately 1.5°C per modification. In these experiments, the T_m values of the oligonucleotides containing 2'-O-methyl modifications were higher than the T_m values of the full deoxy compound of the same sequence.

      Hybridization of "deoxy gap" oligonucleotides to a
15 **structured RNA target:** In further experiments oligonucleotides were hybridized to a larger H-ras target which contains a stable stem loop structure in the codon 12 region. Effects of 2'-O-methyl modifications on antisense hybridization to the structured H-ras target were determined by gel shift analysis
20 as described in Example 7.

      As shown in Figure 9, the full deoxy 17-mer formed the least stable duplex with the hairpin target; the full 2'-O-methyl 17-mer formed the most stable duplex. As deoxy gap size was decreased in these oligonucleotides, increasing the number
25 of 2'-O-methyl residues increased duplex stability.

      Secondary and tertiary structure of the RNA target affects hybridization of antisense oligonucleotides. A series of 11-mer chimeric oligonucleotides were made which hybridize to various regions of the ras hairpin target. ISIS 5055
30 hybridizes to the left side of the stem region (as the hairpin is displayed in Figure 9). ISIS 5056 hybridizes to the left side of the loop. ISIS 5091 hybridizes to the right side of the loop and ISIS 5147 hybridizes to the right side of the stem. All are uniform phosphorothioates with centered 5-deoxy
35 gaps flanked by 2'-O-methyl regions. Only the 11-mer targeted to the left side of the loop bound measurably to the target. The other 11-mers did not bind measurably. Longer versions of

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these oligonucleotides were also made; these 13-mer oligoribonucleotides all demonstrated measurable binding to the hairpin target, with the oligonucleotide targeted to the left side of the loop demonstrating the tightest binding in the gel-

5   shift assay.

RNAse H cleavage directed by deoxy gapped oligonucleotides: Ability of 2'-O-methyl deoxy gap oligonucleotides to direct RNase H cleavage of a complementary RNA was determined *in vitro* using HeLa nuclear extracts as a

10   source of RNase H as described in Example 8. As shown in Figure 10, no cleavage was observed with the fully modified 2'-O-methyl oligonucleotide or one containing a single deoxy residue. Oligonucleotides with a deoxy length of three, four, five, seven or nine were able to direct RNase H cleavage.

15   Deoxy gaps of five, seven or nine are preferred and gaps of seven or nine are most preferred.

Antisense activity of deoxy-gapped oligonucleotides against full length ras mRNA: The beneficial properties of enhanced target affinity conferred by 2'-O-methyl modifications

20   can be exploited for antisense inhibition provided these compounds are equipped with RNase H-sensitive deoxy gaps of the appropriate length. 2'-O-methyl deoxy gap oligonucleotides were tested for antisense activity against the full length H-ras mRNA using the H-ras transactivation reporter gene system

25   described in Example 9. Antisense experiments were performed initially at a single oligonucleotide concentration (100 nM). As shown in Figure 11, chimeric 2'-O-methyl oligonucleotides containing deoxy gaps of five or more residues inhibited H-ras gene expression. These compounds displayed activities greater

30   than that of the full deoxy parent compound.

Dose response experiments were performed using these active compounds, along with the 2'-O-methyl chimeras containing four deoxy residues. As shown in Figure 11B, oligonucleotide-mediated inhibition of full-length H-ras by

35   these oligonucleotides was dose-dependent. The most active compound was the seven-residue deoxy chimera, which displayed

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an activity approximately five times greater than that of the full deoxy oligonucleotide.

Shortened chimeric oligonucleotides: Enhanced target affinity conferred by the 2'-O-methyl modifications was found to confer activity on short chimeric oligonucleotides. A series of short 2'-O-methyl chimeric oligonucleotides were tested for T_m and antisense activity vs. full length ras as described in Example 9. Table 4 shows T_ms for oligonucleotides 11, 13, 15 and 17 nucleotides in length, having deoxy gaps either 5 bases long or 7 bases long. In sharp contrast to the full deoxy 13-mer, both 2'-O-methyl chimeric 13-mers inhibited ras expression, and one of the 11-mers was also active. This is shown in Figure 12.

TABLE 4

LENGTH	T_m (°C)	SEQUENCE	SEQ ID NO:
17	77.2	CCACACCGACGGCGCCC	3
15	69.8	CACACCGACGGCGCC	13
13	62.1	ACACCGACGGCGC	12
11	47.3	CACCGACGGCG	11
17	74.6	CCACACCGACGGCGCCC	3
15	66.2	CACACCGACGGCGCC	13
13	58.0	ACACCGACGGCGC	12
11	27.7	CACCGACGGCG	11

Relative antisense activity and ability to activate RNase H cleavage *in vitro* by chimeric 2'-O-methyl oligonucleotides is well correlated with deoxy length (Figure 13).

Asymmetrical deoxy gaps: It is not necessary that the deoxy gap be in the center of the chimeric molecule. It was found that chimeric molecules having the nucleotides of the region at one end modified at the 2' position to enhance binding and the remainder of the molecule unmodified (2'deoxy) can still inhibit ras expression. Oligonucleotides of SEQ ID NO: 3 (17-mer complementary to mutant codon 12) in which a 7-deoxy gap was located at either the 5' or 3' side of the 17-mer, or at different sites within the middle of the molecule,

- 29 -

all demonstrated RNase H activation and antisense activity. However, a 5-base gap was found to be more sensitive to placement, as some gap positions rendered the duplex a poor activator of RNase H and a poor antisense inhibitor.

5   Therefore, a 7-base deoxy gap is preferred.

        Other sugar modifications: The effects of other 2' sugar modifications besides 2'-O-methyl on antisense activity in chimeric oligonucleotides have been examined.   These modifications are listed in Table 5, along with the T_m values

10 obtained when 17-mer oligonucleotides having 2'-modified nucleotides flanking a 7-base deoxy gap were hybridized with a 25-mer oligoribonucleotide complement as described in Example 6.   A relationship was observed for these oligonucleotides between alkyl length at the 2' position and T_m.   As alkyl

15 length increased, T_m decreased.   The 2'-fluoro chimeric oligonucleotide displayed the highest T_m of the series.

TABLE 5

Correlation of T_m with Antisense Activity
2'-modified 17-mer with 7-deoxy gap
20 CCACACCGACGGCGCCC (SEQ ID NO: 3)

2' MODIFICATION	T_m (°C)	IC50 (nM)
Deoxy	64.2	150
O-Pentyl	68.5	150
O-Propyl	70.4	70
O-Methyl	74.7	20
Fluoro	76.9	10

        These 2' modified oligonucleotides were tested for antisense activity against H-ras using the transactivation reporter gene assay described in Example 9. As shown in Figure

30 14 and Table 5, all of these 2' modified chimeric compounds inhibited ras expression, with the 2'-fluoro 7-deoxy-gap compound the most active. A 2'-fluoro chimeric oligonucleotide with a centered 5-deoxy gap was also active.

        Chimeric phosphorothioate oligonucleotides having SEQ ID

35 NO: 3 having 2'-O-propyl regions surrounding a 5-base or 7-base

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deoxy gap were compared to 2'-O-methyl chimeric oligonucleotides. ras expression in T24 cells was inhibited by both 2'-O-methyl and 2'-O-propyl chimeric oligonucleotides with a 7-deoxy gap and a uniform phosphorothioate backbone. When 5 the deoxy gap was decreased to five nucleotides, only the 2'-O-methyl oligonucleotide inhibited ras expression.

Antisense oligonucleotide inhibition of H-ras gene expression in cancer cells: Two phosphorothioate oligonucleotides (2502, 2503) complementary to the ras AUG 10 region were tested as described in Example 10, along with chimeric oligonucleotides (4998, 5122) having the same sequence and 7-base deoxy gaps flanked by 2'-O-methyl regions. These chimeric oligonucleotides are shown in Table 6.

TABLE 6

15   Chimeric phosphorothioate oligonucleotides
         having 2'-O-methyl ends (bold) and central deoxy gap
                          (AUG target)

OLIGO #	DEOXY	SEQUENCE	SEQ ID NO:
2502	20	CTTATATTCCGTCATCGCTC	1
4998	7	**CTTATATTCCGTCATCGCTC**	1
2503	20	TCCGTCATCGCTCCTCAGGG	2
5122	7	**TCCGTCATCGCTCCTCAGGG**	2

Compound 2503 inhibited ras expression in T24 cells by 71%, and the chimeric compound (4998) inhibited ras mRNA even 25 further (84% inhibition). Compound 2502, also complementary to the AUG region, decreased ras RNA levels by 26% and the chimeric version of this oligonucleotide (5122) demonstrated 15% inhibition. Also included in this assay were two oligonucleotides targeted to the mutant codon 12. Compound 30 2570 (SEQ ID NO: 3) decreased ras RNA by 82% and the 2'-O-methyl chimeric version of this oligonucleotide with a seven-deoxy gap (3985) decreased ras RNA by 95%.

Oligonucleotides 2570 and 2503 were also tested to determine their effects on ras expression in HeLa cells, which 35 have a wild-type (i.e., not activated) H-ras codon 12. While both of these oligonucleotides inhibited ras expression in T24

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cells (having activated codon 12), only the oligonucleotide
(2503) specifically hybridizable with the ras AUG inhibited ras
expression in HeLa cells. Oligonucleotide 2570 (SEQ ID NO: 3),
specifically hybridizable with the activated codon 12, did not
5   inhibit ras expression in HeLa cells, because these cells lack
the activated codon-12 target.

Oligonucleotide   2570,   a   17-mer   phosphorothioate
oligonucleotide   complementary   to   the   codon   12   region   of
activated H-ras, was tested for inhibition of ras expression
10   (as described in Example 10) in T24 cells along with chimeric
phosphorothioate 2'-O-methyl oligonucleotides 3980, 3985 and
3984, which have the same sequence as 2570 and have deoxy gaps
of 5, 7 and 9 bases, respectively (shown in Table 3). The
fully 2'-deoxy oligonucleotide 2570 and the three chimeric
15   oligonucleotides   decreased   ras   mRNA   levels   in   T24   cells.
Compounds 3985 (7-deoxy gap) and 3984 (9-deoxy gap) decreased
ras mRNA by 81%; compound 3980 (5-deoxy gap) decreased ras mRNA
by 61%.   Chimeric oligonucleotides having this sequence, but
having 2'-fluoro-modified nucleotides flanking a 5-deoxy (4689)
20   or 7-deoxy (4690) gap, inhibited ras mRNA expression in T24
cells, with the 7-deoxy gap being preferred (82% inhibition, vs
63% inhibition for the 2'-fluoro chimera with a 5-deoxy gap).

**Antisense oligonucleotide inhibition of proliferation of
cancer cells:** Three 17-mer oligonucleotides having the same
25   sequence (SEQ ID NO: 3), complementary to the codon 12 region
of activated ras, were tested for effects on T24 cancer cell
proliferation as described in Example 11.   3985 has a 7-deoxy
gap flanked by 2'-O-methyl nucleotides, and 4690 has a 7-deoxy
gap flanked by 2'-F nucleotides (all are phosphorothioates).
30   Effects of these oligonucleotides on cancer cell proliferation
correlated well with their effects on ras mRNA expression shown
by Northern blot analysis: oligonucleotide 2570 inhibited cell
proliferation by 61%, the 2'-O-methyl chimeric oligonucleotide
3985 inhibited cell proliferation by 82%, and the 2'-fluoro
35   chimeric analog inhibited cell proliferation by 93%.

In dose-response studies of these oligonucleotides on
cell proliferation, the inhibition was shown to be dose-

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dependent in the 25 nM-100 nM range. IC50 values of 44 nM, 61
nM and 98 nM could be assigned to oligonucleotides 4690, 3985
and 2570, respectively. The random oligonucleotide control had
no effect at the doses tested.

5   The effect of ISIS 2570 on cell proliferation was cell
type-specific. The inhibition of T24 cell proliferation by
this oligonucleotide was four times as severe as the inhibition
of HeLa cells by the same oligonucleotide (100 nM
oligonucleotide concentration). ISIS 2570 is targeted to the
10   activated (mutant) ras codon 12, which is present in T24 but
lacking in HeLa cells, which have the wild-type codon 12.

          Chimeric   backbone-modified   oligonucleotides:
Oligonucleotides discussed in previous examples have had
uniform phosphorothioate backbones. The 2'modified chimeric
15   oligonucleotides discussed above are not active in uniform
phosphodiester backbones. A chimeric oligonucleotide was
synthesized (ISIS 4226) having 2'-O-methyl regions flanking a
5-nucleotide deoxy gap, with the gap region having a P=S
backbone and the flanking regions having a P=O backbone.
20   Another chimeric oligonucleotide (ISIS 4223) having a P=O
backbone in the gap and P=S in flanking regions was also made.
These oligonucleotides are shown in Table 7.

          Additional oligonucleotides were synthesized, completely
2'deoxy and having phosphorothioate backbones containing either
25   a single phosphodiester (ISIS 4248), two phosphodiesters (ISIS
4546), three phosphodiesters (ISIS 4551), four phosphodiesters
(ISIS 4593), five phosphodiesters (ISIS 4606) or ten
phosphodiester linkages (ISIS-4241) in the center of the
molecule. These oligonucleotides are also shown in Table 7.

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TABLE 7

Chimeric backbone (P=S/P=O) oligonucleotides
having 2'-O-methyl ends (bold) and central deoxy gap
(backbone linkages indicated by s (P=S) or o (P=O)
Mutant codon-12 target

OLIGO #	P=S	SEQUENCE	SEQ ID NO:
2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
4226	5	**CoCoAoCoAoCsCsGsAsCsGoGoCoGoCoCoC**	3
4233	11	**CsCsAsCsAsCoCoGoAoCoGsGsCsGsCsCsC**	3
4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4546	14	CsCsAsCsAsCsCsGoAoCsGsGsCsGsCsCsC	3
4551	13	CsCsAsCsAsCsCsGoAoCoGsGsCsGsCsCsC	3
4593	12	CsCsAsCsAsCsCoGoAoCoGsGsCsGsCsCsC	3
4606	11	CsCsAsCsAsCsCoGoAoCoGoGsCsGsCsCsC	3
4241	6	CsCsAsCoAoCoCoGoAoCoGoGoCoGsCsCsC	3

Oligonucleotides were incubated in crude HeLa cellular extracts at 37°C to determine their sensitivity to nuclease degradation as described in Dignam et al., *Nucleic Acids Res.* **1983**, 11, 1475-1489. The oligonucleotide (4233) with a five-diester gap between phosphorothioate/2'-O-methyl wings had a $T_{1/2}$ of 7 hr. The oligonucleotide with a five-phosphorothioate gap in a phosphorothioate/2'-O-methyl molecule had a $T_{1/2}$ of 30 hours. In the set of oligonucleotides having one to ten diester linkages, the oligonucleotide (4248) with a single phosphodiester linkage was as stable to nucleases as was the full-phosphorothioate molecule, ISIS 2570, showing no degradation after 5 hours in HeLa cell extract. Oligonucleotides with two-, three and four-diester gaps had $T_{1/2}$ of approximately 5.5 hours, 3.75 hours, and 3.2 hours, and oligonucleotides with five or ten deoxy linkages had $T_{1/2}$ of 1.75 hours and 0.9 hours, respectively.

Antisense activity of chimeric backbone-modified oligonucleotides: A uniform phosphorothioate backbone is not required for antisense activity. ISIS 4226 and ISIS 4233 were tested in the ras-luciferase reporter system for effect on ras expression as described in Examples 2-5, along with ISIS 2570

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(fully phosphorothioate/all deoxy), ISIS 3980 (fully
phosphorothioate, 2'-O-methyl wings with deoxy gap) and ISIS
3961 (fully phosphodiester, 2'-O-methyl wings with deoxy gap).
All of the oligonucleotides having a P=S (i.e., nuclease-

5   resistant) gap region inhibited ras expression. This is shown
in Figure 15. The two completely 2'deoxy oligonucleotides
having phosphorothioate backbones containing either a single
phosphodiester (ISIS 4248) or ten phosphodiester linkages (ISIS
4241) in the center of the molecule were also assayed for

10 activity. The compound containing a single P=O was just as
active as a full P=S molecule, while the same compound
containing ten P=O was completely inactive.

    Chimeric phosphorothioate oligonucleotides of SEQ ID NO:
3 were made, having a phosphorothioate backbone in the 7-base

15 deoxy gap region only, and phosphodiester in the flanking
regions, which were either 2'-O-methyl or 2'-O-propyl. The
oligonucleotide with the 2'-O-propyl diester flanking regions
was able to inhibit ras expression.

    Inhibition of ras-luciferase gene expression by antisense

20 **oligonucleotides containing modified bases:** A series of
antisense phosphorothioate oligonucleotides complementary to
the codon-12 point mutation of activated ras were synthesized
as described, having a 2-(amino)adenine at the position
complementary to the uracil of the mutated codon 12. Because

25 the amino group at the 2-position of the adenine is able to
hydrogen bond with the oxygen in the 2-position on the uracil,
three hydrogen bonds instead of the usual two are formed. This
serves to greatly stabilize the hybridization of the 2-
(amino)adenine-modified antisense oligonucleotide to the

30 activated ras gene, while destabilizing or having no net effect
on the stability of this oligonucleotide to the wild-type codon
12, because of the modified A-G mismatch at this position.
This increases the specificity of the modified oligonucleotide
for the desired target.

35   An oligonucleotide having a single 2,6-(diamino)adenosine
at this position in an otherwise unmodified uniform
phosphorothioate 17-mer (sequence identical to 2570, SEQ ID NO:

- 35 -

3) was found to be at least as effective an RNase H substrate as the 2570 sequence. It is therefore expected to be an effective antisense molecule. An oligonucleotide having a single 2,-(diamino)adenosine at this position in a deoxy gapped
5 phosphorothioate oligonucleotide of the same sequence also demonstrates RNase H activation.

in vivo anti-tumor data: ISIS 2503 (SEQ ID NO: 2) has been evaluated for activity against human tumors *in vivo* as described in Examples 13 and 14. These studies employed a
10 human lung adenocarcinoma cell line (A549) which was subcutaneously implanted into nude mice, resulting in tumor growth at site of implantation. Since these cells do not contain a mutation in the Ha-ras gene, but do express normal Ha-ras, only the AUG-directed oligonucleotide ISIS 2503 was
15 evaluated for anti-tumor activity.

In the first study, phosphorothioate oligonucleotides in saline were administered by intraperitoneal injection at a dosage of 20 mg/kg. Drug treatment was initiated at the time tumors first became visible (28 days following tumor cell
20 inoculation) and treatments were performed every other day. As shown in Figure 16, no effect on tumor growth was observed after treatment with the unrelated control phosphorothioate oligonucleotide ISIS 1082. However, significant inhibition of tumor growth was observed for the Ha-ras-specific
25 oligonucleotide ISIS 2503 (SEQ ID NO: 2). The anti-tumor effects of the Ha-ras compound were first observed 20 days following initiation of drug treatment and continued throughout the duration of the study.

In a second study, phosphorothioate oligonucleotides were
30 prepared in a cationic lipid formulation (DMRIE:DOPE) and administered by subcutaneous injection as described in Example 15. Drug treatment was initiated one week following tumor cell inoculation and was performed three times a week for only four weeks. As was observed in the first study, administration of
35 the Ha-ras-specific compound ISIS 2503 (SEQ ID NO: 2) caused a marked reduction in tumor growth whereas the unrelated control oligonucleotide (ISIS 1082) had no significant effect (Figure

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17). Reduction in tumor volume was first observed 20 days following appearance of visible tumors and continued over time throughout the remainder of the study.

Stability of 2'-modified phosphodiester oligonucleotides

5 **in cells:** Modification of oligonucleotides to confer nuclease stability is required for antisense activity in cells. Certain modifications at the 2' position of the sugar have been found to confer nuclease resistance sufficient to elicit antisense effects in cells without any backbone modification. As shown

10 in Figure 18, a uniformly 2'-propoxy modified phosphodiester oligonucleotide (SEQ ID NO: 3) was found to inhibit Ha-ras expression in T24 cells, 24 hours after administration, at a level equivalent to a phosphorothioate 2'-deoxyoligonucleotide having the same sequence. Uniform 2'-methoxy phosphodiester

15 oligonucleotide also showed some activity.   2'-pentoxy modifications were found to be at least as active as the 2'-propoxy.

Antisense oligonucleotides active against Ki-ras:

Oligonucleotides were designed to be complementary to the

20 5'-untranslated region, 3'-untranslated region and coding region of the human Ki-ras oncogene. McGrath, J.P. et al., (1983) *Nature* 304, 501-506. Of the latter, oligonucleotides were targeted to codons 12 and 61 which are known sites of mutation that lead to Ki-ras-mediated transformation, and also

25 to codon 38, which is not known to be involved in transformation. The oligonucleotides are shown in Table 8.

Table 8

Antisense Oligonucleotides Complementary to Human Ki-ras

ISIS #	SEQUENCE	TARGET	SEQ ID NO:
30 6958	CTG CCT CCG CCG CCG CGG CC	5' UTR/5' cap	20
6957	CAG TGC CTG CGC CGC GCT CG	5'-UTR	21
6956	AGG CCT CTC TCC CGC ACC TG	5'-UTR	22
6953	TTC AGT CAT TTT CAG CAG GC	AUG	23
6952	TTA TAT TCA GTC ATT TTC AG	AUG	24
35 6951	CAA GTT TAT ATT CAG TCA TT	AUG	25
6950	GCC TAC GCC ACC AGC TCC AAC	Codon 12 (WT)	26
6949	CTA CGC CAC CAG CTC CA	Codon 12 (WT)	27
6948	G TAC TCC TCT TGA CCT GCT GT	Codon 61 (WT)	28
6947	CCT GTA GGA ATC CTC TAT TGT	Codon 38	29
40 6946	GGT AAT GCT AAA ACA AAT GC	3'-UTR	30
6945	GGA ATA CTG GCA CTT CGA GG	3'-UTR	31

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7453   TAC GCC AAC AGC TCC              Codon 12 (G→T mut.) 32
7679   TTT TCA GCA GGC CTC TCT CC       5'-UTR/AUG          33
```

Twelve Ki-ras-specific oligonucleotides were screened for antisense activity against three colon carcinoma cell lines 5 that contain a mutation at codon 12 in the Ki-ras oncogene and evaluated by measurement of Ki-ras mRNA levels. As shown in Figure 19, half of the tested compounds displayed significant activity (at least 40% inhibition) against the Ki-ras transcript, with the most active compounds being targeted to 10 the 5'- and 3'-untranslated regions. However, significant inhibition of Ki-ras expression was also observed for compounds directed against wild type codons 12 and 61. Compounds that displayed significant activity were effective against all three carcinoma cell lines tested.

15   Dose response analysis of these compounds demonstrated that ISIS 6958 and ISIS 6957, both of which target the 5'-UTR, are the most potent inhibitors of Ki-ras in this series of oligonucleotides. These oligonucleotides were examined for their ability to inhibit proliferation of Ki-ras transformed 20 cell lines. The colon carcinoma cell line SW480 was treated with a single dose of oligonucleotide (200 nM) and cell number was determined over a five-day period. As shown in Figure 20, both Ki-ras specific oligonucleotides were effective inhibitors of proliferation of SW480 cells, with ISIS 6957 (SEQ ID NO: 21) 25 showing greater activity than ISIS 6958 (SEQ ID NO: 20). This difference in activity correlates well with the inhibition of Ki-ras mRNA expression (Figure 19).

Selectivity of inhibition of mutant Ki-ras relative to normal Ki-ras: Oligonucleotides targeted to Ki-ras have been 30 examined for their ability to selectively inhibit mutant Ki-ras relative to normal Ki-ras. Two cell lines were employed: the SW480 cell line that expresses mutant Ki-ras (codon 12, G to T transversion) and a cell line (HeLa) that expresses normal Ki-ras. Two oligonucleotides were tested: ISIS 6957, SEQ ID NO: 35 21, a 20mer phosphorothioate targeted to the 5'-untranslated region of Ki-ras, and ISIS 7453, SEQ ID NO: 32, a 15mer phosphorothioate targeted to the Ki-ras codon 12 region. Ki-

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ras mRNA levels were measured 24 hours after treatment. The codon 12-directed compound was effective in the cell line expressing mutant Ki-ras. However, as shown in Figure 21, the Ki-ras oligonucleotide targeted to the 5'-untranslated region 5 was a potent inhibitor of Ki-ras expression in both cell lines. Selectivity for mutant Ki-ras was found to be dependent on oligonucleotide concentration and affinity for the RNA target.

Ki-ras oligonucleotides with deoxy gaps: Phosphorothioate oligonucleotides (SEQ ID NO: 21, targeted to the 5'- 10 untranslated region of Ki-ras) were synthesized with 2'-O-methyl modifications flanking central 2'-deoxy gap regions of 6 or 8 nucleotides in length. Both gapped oligonucleotides were active against Ki-ras expression as determined by Northern blot analysis. A uniformly 2'-O-methylated compound (no deoxy 15 gap) was inactive. An additional oligonucleotide, ISIS 7679 (SEQ ID NO: 33, complementary to the 5' untranslated/AUG region of Ki-ras), was also found to be active when synthesized with a 6- or 8- nucleotide deoxy gap.

The oligonucleotides used in accordance with this 20 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 Applied Biosystems. Any other means for such synthesis may also be employed, however the actual synthesis of the oligonucleotides 25 are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

The oligonucleotides of this invention are designed to 30 be complementary to, and thus hybridizable with, messenger RNA derived from the H-ras gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a loss of its function in the cell. The functions of messenger RNA to be interfered with include all vital 35 functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and

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possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to interfere with expression of the H-ras gene. Some oligonucleotides of this invention are designed to activate RNAse H cleavage of the ras mRNA.

The protein products of the other mammalian ras genes, N-ras and K-ras, are identical to H-ras over the first 85 amino acids. The nucleic acid sequences of the three ras genes, while not identical, are known, and persons of ordinary skill in the art will be able to use this invention as a guide in preparing oligonucleotides specifically hybridizable with the N-ras and K-ras genes. While the preferred embodiments of this invention relate to antisense oligonucleotides specifically hybridizable with codon 12 of the H-ras mRNA, this invention can be used by persons skilled in the art as a guide in preparing oligonucleotides specifically hybridizable with other point mutations of the ras gene, particularly the well defined point mutations at codon 12, codon 13 and codon 61 of H-ras, N-ras and K-ras, the sequences of which are known.

The oligonucleotides of this invention can be used in diagnostics, therapeutics and as research reagents and kits. Since the oligonucleotides of this invention hybridize to the ras gene, sandwich and other assays can easily be constructed to exploit this fact. Furthermore, since the oligonucleotides of this invention hybridize preferentially to the mutant (activated) form of the ras oncogene, such assays can be devised for screening of cells and tissues for ras conversion from wild-type to activated form. Such assays can be utilized for differential diagnosis of morphologically similar tumors, and for detection of increased risk of cancer stemming from ras gene activation. Provision of means for detecting hybridization of oligonucleotide with the ras gene can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of ras or activated ras may also be prepared.

The following examples illustrate the present invention

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and are not intended to limit the same.

EXAMPLES

Example 1   Oligonucleotide Synthesis

     Substituted and unsubstituted deoxyoligonucleotides were
5 synthesized on an automated DNA synthesizer (Applied Biosystems
model 380B) using standard phosphoramidate chemistry with
oxidation by iodine.   For phosphorothioate oligonucleotides,
the standard oxidation bottle was replaced by 0.2 M solution of
3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the
10 stepwise thiation of the phosphite linkages.   The thiation wait
step was increased to 68 sec and was followed by the capping
step.   After cleavage from the CPG column and deblocking in
concentrated   ammonium   hydroxide   at   55°C   (18   hr),   the
oligonucleotides were purified by precipitation twice out of
15 0.5 M NaCl solution with 2.5 volumes ethanol.   Analytical gel
electrophoresis was accomplished in 20% acrylamide, 8 M urea,
454 mM Tris-borate buffer, pH=7.0.   Oligonucleotides were
judged from polyacrylamide gel electrophoresis to be greater
than 80% full-length material.
20   Oligoribonucleotides were synthesized using the automated
synthesizer and 5'-dimethoxy-trityl 2'-*tert*-butyldimethylsilyl
3'-O-phosphoramidites (American Bionetics, Hayward, CA).   The
protecting group on the exocyclic amines of A,C and G was
phenoxyacetyl [Wu, T., Oglivie, K.K., and Pon, R.T., *Nucl.*
25 *Acids Res.* 1989, 17, 3501-3517].   The standard synthesis cycle
was modified by increasing the wait step after the pulse
delivery of tetrazole to 900 seconds.   Oligonucleotides were
deprotected by overnight incubation at room temperature in
methanolic ammonia.   After drying *in vacuo*, the 2'-silyl group
30 was removed by overnight incubation at room temperature in 1 M
tetrabutylammoniumfluoride   (Aldrich;   Milwaukee,   WI)   in
tetrahydrofuran.   Oligonucleotides were purified using a C-18
Sep-Pak cartridge (Waters; Milford, MA) followed by ethanol
precipitation.   Analytical   denaturing   polyacrylamide
35 electrophoresis demonstrated the RNA oligonucleotides were
greater than 90% full length material.

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Example 2   **ras-Luciferase Reporter Gene Assembly**

The ras-luciferase reporter genes described in this study
were assembled using PCR technology. Oligonucleotide primers
were synthesized for use as primers for PCR cloning of the 5'-
5   regions of exon 1 of both the mutant (codon 12) and non-mutant
(wild-type) human H-ras genes.   The plasmids pT24-C3,
containing the c-H-ras1 activated oncogene (codon 12, GGC→GTC),
and pbc-N1, containing the c-H-ras proto-oncogene, were
obtained from the American Type Culture Collection (Bethesda,
10   MD).   The plasmid pT3/T7 luc, containing the 1.9 kb firefly
luciferase gene, was obtained from Clontech Laboratories (Palo
Alto, CA).   The oligonucleotide PCR primers were used in
standard PCR reactions using mutant and non-mutant H-ras genes
as templates. These primers produce a DNA product of 145 base
15   pairs corresponding to sequences -53 to +65 (relative to the
translational initiation site) of normal and mutant H-ras,
flanked by NheI and HindIII restriction endonuclease sites.
The PCR product was gel purified, precipitated, washed and
resuspended in water using standard procedures.
20   PCR primers for the cloning of the *P. pyralis* (firefly)
luciferase gene were designed such that the PCR product would
code for the full-length luciferase protein with the exception
of the amino-terminal methionine residue, which would be
replaced with two amino acids, an amino-terminal lysine residue
25   followed by a leucine residue. The oligonucleotide PCR primers
used for the cloning of the luciferase gene were used in
standard PCR reactions using a commercially available plasmid
(pT3/T7-Luc) (Clontech), containing the luciferase reporter
gene, as a template.   These primers yield a product of
30   approximately 1.9 kb corresponding to the luciferase gene,
flanked by unique HindIII and BssHII restriction endonuclease
sites.   This fragment was gel purified, precipitated, washed
and resuspended in water using standard procedures.
To complete the assembly of the ras-luciferase fusion
35   reporter gene, the ras and luciferase PCR products were
digested with the appropriate restriction endonucleases and
cloned by three-part ligation into an expression vector

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containing the steroid-inducible mouse mammary tumor virus
promotor MMTV using the restriction endonucleases NheI, HindIII
and BssHII. The resulting clone results in the insertion of H-
ras 5' sequences (-53 to +65) fused in frame with the firefly
5   luciferase gene. The resulting expression vector encodes a
ras-luciferase fusion product which is expressed under control
of the steroid-inducible MMTV promoter. These plasmid
constructions contain sequences encoding amino acids 1-22 of
activated (RA2) or normal (RA4) H-ras proteins fused in frame
10   with sequences coding for firefly luciferase. Translation
initiation of the ras-luciferase fusion mRNA is dependent upon
the natural H-ras AUG codon. Both mutant and normal H-ras
luciferase fusion constructions were confirmed by DNA sequence
analysis using standard procedures.

15   **Example 3   Transfection of Cells with Plasmid DNA**
        Transfections were performed as described by Greenberg,
M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel,
R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman
and K. Strahl, eds.), John Wiley and Sons, NY, with the
20   following modifications. HeLa cells were plated on 60 mm
dishes at 5×10^5 cells/dish. A total of 10 µg or 12 µg of DNA
was added to each dish, of which 1 µg was a vector expressing
the rat glucocorticoid receptor under control of the
constitutive Rous sarcoma virus (RSV) promoter and the
25   remainder was ras-luciferase reporter plasmid. Calcium
phosphate-DNA coprecipitates were removed after 16-20 hours by
washing with Tris-buffered saline [50 Mm Tris-Cl (pH 7.5), 150
mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with
10% fetal bovine serum was then added to the cells. At this
30   time, cells were pre-treated with antisense oligonucleotides
prior to activation of reporter gene expression by
dexamethasone.

Example 4   Oligonucleotide Treatment of Cells
        Following plasmid transfection, cells were washed with
35   phosphate buffered saline prewarmed to 37°C and Opti-MEM

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containing   5   µg/mL   N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-
trimethylammonium chloride (DOTMA) was added to each plate (1.0
ml per well). Oligonucleotides were added from 50 µM stocks to
each plate and incubated for 4 hours at 37°C. Medium was
5 removed and replaced with DMEM containing 10% fetal bovine
serum and the appropriate oligonucleotide at the indicated
concentrations and cells were incubated for an additional 2
hours at 37°C before reporter gene expression was activated by
treatment of cells with dexamethasone to a final concentration
10 of 0.2 µM. Cells were harvested and assayed for luciferase
activity fifteen hours following dexamethasone stimulation.

Example 5   Luciferase Assays
      Luciferase was extracted from cells by lysis with the
detergent Triton X-100 as described by Greenberg, M.E., in
15 *Current Protocols in Molecular Biology*, (F.M. Ausubel, R.
Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and
K. Strahl, eds.), John Wiley and Sons, NY. A Dynatech ML1000
luminometer was used to measure peak luminescence upon addition
of luciferin (Sigma) to 625 µM. For each extract, luciferase
20 assays were performed multiple times, using differing amounts
of extract to ensure that the data were gathered in the linear
range of the assay.

Example 6   Melting Curves
      Absorbance vs temperature curves were measured at 260 nm
25 using a Gilford 260 spectrophotometer interfaced to an IBM PC
computer and a Gilford Response II spectrophotometer. The
buffer contained 100 mM Na^+, 10 mM phosphate and 0.1 mM EDTA,
pH 7. Oligonucleotide concentration was 4 µM each strand
determined from the absorbance at 85°C and extinction
30 coefficients calculated according to Puglisi and Tinoco,
Methods in Enzymol. 1989, 180, 304-325. T_m values, free
energies of duplex formation and association constants were
obtained from fits of data to a two state model with linear
sloping baselines.   Petersheim, M. and Turner, D.H.,
35 *Biochemistry* 1983, 22, 256-263. Reported parameters are

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averages of at least three experiments. For some
oligonucleotides, free energies of duplex formation were also
obtained from plots of T_m^{-1} vs $\log_{10}$ (concentration). Borer,
P.N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O.C., *J.*
5 *Mol. Biol.*, 1974, 86, 843-853.

Example 7   Gel Shift Assay

      The structured ras target transcript, a 47-nucleotide
hairpin containing the mutated codon 12, was prepared and
mapped as described in Lima et al., *Biochemistry* 1991, 31,
10 12055-12061. Hybridization reactions were prepared in 20 µl
containing 100 mM sodium, 10 mM phosphate, 0.1 mM EDTA, 100 CPM
of *T7*-generated RNA (approximately 10 pM), and antisense
oligonucleotide ranging in concentration from 1 pM to 10 µM.
Reactions were incubated 24 hours at 37°C. Following
15 hybridization, loading buffer was added to the reactions and
reaction products were resolved on 20% native polyacrylamide
gels, prepared using 45 mM tris-borate and 1 mM $MgCl_2$ (TBM).
Electrophoresis was carried out at 10°C and gels were
quantitated using a Molecular Dynamics Phosphorimager.

20 **Example 8   RNase H Analysis**

      RNase H assays were performed using a chemically
synthesized 25-base oligoribonucleotide corresponding to bases
+23 to +47 of activated (codon 12, G→U) H-ras mRNA. The 5'
end-labeled RNA was used at a concentration of 20 nM and
25 incubated with a 10-fold molar excess of antisense
oligonucleotide in a reaction containing 20 mM tris-Cl, pH 7.5,
100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 µg tRNA and 4
U RNasin in a final volume of 10 µl. The reaction components
were preannealed at 37°C for 15 minutes then allowed to cool
30 slowly to room temperature. HeLa cell nuclear extracts were
used as a source of mammalian RNase H. Reactions were
initiated by addition of 2 µg of nuclear extract (5 µl) and
reactions were allowed to proceed for 10 minutes at 37°C.
Reactions were stopped by phenol/chloroform extraction and RNA
35 components were precipitated with ethanol. Equal CPMs were

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loaded on a 20% polyacrylamide gel containing 7M urea and RNA
cleavage products were resolved and visualized by
electrophoresis followed by autoradiography. Quantitation of
cleavage products was performed using a Molecular Dynamics
5 Densitometer.

Example 9   ras Transactivation Reporter Gene System

The expression plasmid pSV2-oli, containing an activated
(codon 12, GGC→GTC) H-ras cDNA insert under control of the
constitutive SV40 promoter, was a gift from Dr. Bruno Tocque
10 (Rhone-Poulenc Sante, Vitry, France). This plasmid was used as
a template to construct, by PCR, a H-ras expression plasmid
under regulation of the steroid-inducible mouse mammary tumor
virus (MMTV) promoter. To obtain H-ras coding sequences, the
570 bp coding region of the H-ras gene was amplified by PCR.
15 The PCR primers were designed with unique restriction
endonuclease sites in their 5'-regions to facilitate cloning.
The PCR product containing the coding region of the H-ras codon
12 mutant oncogene was gel purified, digested, and gel purified
once again prior to cloning. This construction was completed
20 by cloning the insert into the expression plasmid pMAMneo
(Clontech Laboratories, CA).

The ras-responsive reporter gene pRDO53 was used to
detect ras expression. Owen et al., *Proc. Natl. Acad. Sci.*
U.S.A. 1990, 87, 3866-3870.

25 **Example 10   Northern blot analysis of ras**
                    expression *in vivo*

The human urinary bladder cancer cell line T24 was
obtained from the American Type Culture Collection (Rockville
MD). Cells were grown in McCoy's 5A medium with L-glutamine
30 (Gibco BRL, Gaithersburg MD), supplemented with 10% heat-
inactivated fetal calf serum and 50 U/ml each of penicillin and
streptomycin. Cells were seeded on 100 mm plates. When they
reached 70% confluency, they were treated with oligonucleotide.
Plates were washed with 10 ml prewarmed PBS and 5 ml of Opti-
35 MEM reduced-serum medium containing 2.5 μl DOTMA.

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Oligonucleotide was then added to the desired concentration. After 4 hours of treatment, the medium was replaced with McCoy's medium.   Cells were harvested 48 hours after oligonucleotide treatment and RNA was isolated using a standard

5 CsCl purification method. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY.

The human epithelioid carcinoma cell line HeLa 229 was
10 obtained from the American Type Culture Collection (Bethesda, MD). HeLa cells were maintained as monolayers on 6-well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. Treatment with oligonucleotide and isolation of RNA were essentially as
15 described above for T24 cells.

Northern hybridization: 10 µg of each RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred overnight to GeneBind 45 nylon membrane (Pharmacia LKB, Piscataway, NJ) using standard methods. Kingston, R.E.,
20 in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY.   RNA was UV-crosslinked to the membrane. Double-stranded ^{32}P-labeled probes were synthesized using the Prime a Gene labeling kit (Promega,
25 Madison WI). The ras probe was a SalI-NheI fragment of a cDNA clone of the activated (mutant) H-ras mRNA having a GGC-to-GTC mutation at codon-12. The control probe was G3PDH. Blots were prehybridized for 15 minutes at 68°C with the QuickHyb hybridization solution (Stratagene, La Jolla, CA). The heat-
30 denatured radioactive probe (2.5 x 10^6 counts/2 ml hybridization solution) mixed with 100 µl of 10 mg/ml salmon sperm DNA was added and the membrane was hybridized for 1 hour at 68°C. The blots were washed twice for 15 minutes at room temperature in 2x SSC/0.1% SDS and once for 30 minutes at 60°C
35 with 0.1XSSC/0.1%SDS. Blots were autoradiographed and the intensity of signal was quantitated using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Northern

blots were first hybridized with the ras probe, then stripped
by boiling for 15 minutes in 0.1x SSC/0.1%SDS and rehybridized
with the control G3PDH probe to check for correct sample
loading.

5 **Example 11**   **Antisense oligonucleotide inhibition of**
                      proliferation of cancer cells

     Cells were cultured and treated with oligonucleotide
essentially as described in Example 10. Cells were seeded on
60 mm plates and were treated with oligonucleotide in the
10 presence of DOTMA when they reached 70% confluency. Time
course experiment: On day 1, cells were treated with a single
dose of oligonucleotide at a final concentration of 100 nM. The
growth medium was changed once on day 3 and cells were counted
every day for 5 days, using a counting chamber. Dose-response
15 experiment: Various concentrations of oligonucleotide (10, 25,
50, 100 or 250 nM) were added to the cells and cells were
harvested and counted 3 days later. Oligonucleotides 2570,
3985 and 4690 were tested for effects on T24 cancer cell
proliferation.

20 **Example 12**   **Synthesis   of   2-(amino)adenine-substituted**
                      oligonucleotides

     Oligonucleotides are synthesized as in Example 1, with
the following exception: at positions at which a 2-
(amino)adenine is desired, the standard phosphoramidite is
25 replaced with a commercially available 2-aminodeoxyadenosine
phosphoramidite (Chemgenes).

    Example 13   **Culture of A549 cells**

         A549 cells (obtained from the American Type Culture
Collection, Bethesda MD) were grown to confluence in 6-well
30 plates (Falcon Labware, Lincoln Park, NJ) in Dulbecco's
modified Eagle's medium (DME) containing 1 g glucose/liter and
10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA).

    Example 14   **Oligonucleotide treatment of human tumor cells**

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in nude mice - intraperitoneal injection

Human lung carcinoma A549 cells were harvested and 5 x 10^6 cells (200 µl)were injected subcutaneously into the inner thigh of nude mice. Palpable tumors develop in approximately

5   one month. Phosphorothioate oligonucleotides ISIS 2503 and 1082 (unrelated control) were administered to mice intraperitoneally at a dosage of 20 mg/kg body weight, every other day for approximately ten weeks. Mice were monitored for tumor growth during this time.

10 **Example 15**   **Oligonucleotide treatment of human tumor cells in nude mice - subcutaneous injection with cationic lipid**

Human lung carcinoma A549 cells were harvested and 5 x 10^6 cells (200 µl) were injected subcutaneously into the inner

15 thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and the unrelated control oligonucleotide 1082 (dosage 5 mg/kg), prepared in a cationic lipid formulation (DMRIE/DOPE, 60 mg/kg) were administered to mice subcutaneously at the tumor site.

20 Drug treatment began one week following tumor cell inoculation and was given twice a week for only four weeks. Mice were monitored for tumor growth for a total of nine weeks.

Example 16   **Stability of 2' modified oligonucleotides in T24 cells**

25   T24 bladder cancer cells were grown as described in Example 10. Cells were treated with a single dose (1 µM) of oligonucleotide and assayed for Ha-ras mRNA expression by Northern blot analysis 24 hours later. Oligonucleotides tested were analogs of ISIS 2570 (SEQ ID NO: 3), a 17mer targeted to

30 Ha-ras codon 12.

Example 17   **Activity of Ki-ras oligonucleotides against three colon carcinoma cell lines**

Human colon carcinoma cell lines Calu 1, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC)

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and cultured and maintained as described for HeLa cells in
Example 10.   Cells were treated with a single dose of
oligonucleotide (200 mM) and Ki-ras mRNA expression was
measured by Northern blot analysis 24 hours later.   For
5 proliferation studies, cells were treated with a single dose of
oligonucleotide (200 nM) at day zero and cell number was
monitored over a five-day period.

**Example 18   Oligonucleotide inhibition of mutant vs. wild-
type Ki-ras**

10   SW480 cells were cultured as in the previous example.
HeLa cells were cultured as in Example 10.   Cells were treated
with a single dose (100 nM) of oligonucleotide and mRNA levels
were determined by Northern blot analysis 24 hours later.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Monia et al.

    (ii) TITLE OF INVENTION: Antisense Oligonucleotide
        Inhibition of the ras Gene

    (iii) NUMBER OF SEQUENCES: 33

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    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

        (B) COMPUTER: IBM PS/2

        (C) OPERATING SYSTEM: PC-DOS

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(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

CTTATATTCC GTCATCGCTC   20

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

TCCGTCATCG CTCCTCAGGG   20

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(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 17

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

CCACACCGAC GGCGCCC   17

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 19

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

CCCACACCGA CGGCGCCCA   19

(2) INFORMATION FOR SEQ ID NO: 5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 21

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

GCCCACACCG ACGGCGCCCA C   21

(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 23

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

    TGCCCACACC GACGGCGCCC ACC   23

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

    TATTCCGTCA TCGCTCCTCA   20

(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 5

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

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

    CGACG   5

(2) INFORMATION FOR SEQ ID NO: 9:

      (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 7

          (B) TYPE: Nucleic Acid

          (C) STRANDEDNESS: Single

          (D) TOPOLOGY: Linear

      (iv) ANTI-SENSE: Yes

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGACGG   7

(2) INFORMATION FOR SEQ ID NO: 10:

      (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 9

          (B) TYPE: Nucleic Acid

          (C) STRANDEDNESS: Single

          (D) TOPOLOGY: Linear

      (iv) ANTI-SENSE: Yes

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCGACGGC   9

(2) INFORMATION FOR SEQ ID NO: 11:

      (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 11

          (B) TYPE: Nucleic Acid

          (C) STRANDEDNESS: Single

          (D) TOPOLOGY: Linear

      (iv) ANTI-SENSE: Yes

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CACCGACGGC G   11

(2) INFORMATION FOR SEQ ID NO: 12:

      (i) SEQUENCE CHARACTERISTICS:

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                    (A) LENGTH: 13

                    (B) TYPE: Nucleic Acid

                    (C) STRANDEDNESS: Single

                    (D) TOPOLOGY: Linear

            (iv) ANTI-SENSE: Yes

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

        ACACCGACGG CGC   13

    (2) INFORMATION FOR SEQ ID NO: 13:

        (i) SEQUENCE CHARACTERISTICS:

                    (A) LENGTH: 15

                    (B) TYPE: Nucleic Acid

                    (C) STRANDEDNESS: Single

                    (D) TOPOLOGY: Linear

            (iv) ANTI-SENSE: Yes

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

        CACACCGACG GCGCC   15

    (2) INFORMATION FOR SEQ ID NO: 14:

        (i) SEQUENCE CHARACTERISTICS:

                    (A) LENGTH: 16

                    (B) TYPE: Nucleic Acid

                    (C) STRANDEDNESS: Single

                    (D) TOPOLOGY: Linear

            (iv) ANTI-SENSE: Yes

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

        CCACACCGAC GGCGCC   16

    (2) INFORMATION FOR SEQ ID NO: 15:

        (i) SEQUENCE CHARACTERISTICS:

                    (A) LENGTH: 16

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        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CACACCGACG GCGCCC   16

(2) INFORMATION FOR SEQ ID NO: 16:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCACACCGA CGGCGCCC   18

(2) INFORMATION FOR SEQ ID NO: 17:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCACACCGAC GGCGCCCA   18

(2) INFORMATION FOR SEQ ID NO: 18:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 25

        (B) TYPE: Nucleic Acid

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      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTGCCCACAC CGACGGCGCC CACCA   25

(2) INFORMATION FOR SEQ ID NO: 19:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 17

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCACACCGCC GGCGCCC   17

(2) INFORMATION FOR SEQ ID NO: 20:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 20

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

      CTGCCTCCGC CGCCGCGGCC   20

(2) INFORMATION FOR SEQ ID NO: 21:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 20

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

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            (D) TOPOLOGY: Linear

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

            CAGTGCCTGC GCCGCGCTCG   (20)

(2) INFORMATION FOR SEQ ID NO: 22:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 20

            (B) TYPE: Nucleic Acid

            (C) STRANDEDNESS: Single

            (D) TOPOLOGY: Linear

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

            AGGCCTCTCT CCCGCACCTG   (20)

(2) INFORMATION FOR SEQ ID NO: 23:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 20

            (B) TYPE: Nucleic Acid

            (C) STRANDEDNESS: Single

            (D) TOPOLOGY: Linear

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

            TTCAGTCATT TTCAGCAGGC   (20)

(2) INFORMATION FOR SEQ ID NO: 24:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 20

            (B) TYPE: Nucleic Acid

            (C) STRANDEDNESS: Single

            (D) TOPOLOGY: Linear

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(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

   TTATATTCAG TCATTTTCAG   (20)

(2) INFORMATION FOR SEQ ID NO: 25:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 20

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

      CAAGTTTATA TTCAGTCATT   (20)

(2) INFORMATION FOR SEQ ID NO: 26:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 21

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

      GCCTACGCCA CCAGCTCCAA C (21)

(2) INFORMATION FOR SEQ ID NO: 27:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 17

      (B) TYPE: Nucleic Acid

      (C) STRANDEDNESS: Single

      (D) TOPOLOGY: Linear

   (iv) ANTI-SENSE: Yes

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CTACGCCACC AGCTCCA (17)

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTACTCCTCT TGACCTGCTG T (21)

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCTGTAGGAA TCCTCTATTG T (21)

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

- 61 -

        GGTAATGCTA AAACAAATGC   (20)

(2) INFORMATION FOR SEQ ID NO: 31:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

        GGAATACTGG CACTTCGAGG   (20)

(2) INFORMATION FOR SEQ ID NO: 32:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 15

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

        TACGCCAACA GCTCC   (15)

(2) INFORMATION FOR SEQ ID NO: 33:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: Nucleic Acid

        (C) STRANDEDNESS: Single

        (D) TOPOLOGY: Linear

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

        TTTTCAGCAG GCCTCTCTCC   (20)

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CLAIMS

What is claimed is:

1.   An oligonucleotide comprising from 8 to 30
nucleotide units specifically hybridizable with selected DNA or
5 mRNA deriving from the human H-ras gene.

2.   The oligonucleotide of claim 1 specifically
hybridizable with a translation initiation site or codon 12 of
the human H-ras gene.

3.   The oligonucleotide of claim 1 in a pharmaceutically
10 acceptable carrier.

4.   The oligonucleotide of claim 1 wherein at least one
of the linking groups between nucleotide units comprises a
sulfur-containing modification.

5.   The oligonucleotide of claim 4 wherein the sulfur-
15 containing modification is a phosphorothioate modification.

6.   The oligonucleotide of claim 1 wherein at least one
of the nucleotide units of the oligonucleotide is modified at
the 2' position.

7.   The oligonucleotide of claim 6 wherein said
20 modification at the 2' position is a 2'-O-alkyl or a 2'-fluoro
modification.

8.   An oligonucleotide comprising from 8 to 30
nucleotide units specifically hybridizable with selected DNA or
mRNA deriving from the human H-ras gene, being a substrate for
25 RNAse H and having at least one modification to enhance
nuclease resistance and at least one modification to enhance
affinity for said ras DNA or mRNA.

9.   An oligonucleotide comprising SEQ ID NO: 2 or SEQ

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ID NO: 3.

10.   A chimeric oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the ras gene, being stabilized against nucleases and containing a first region having at least one nucleotide which is modified to enhance target affinity and a second region which is a substrate for RNAse H.

11.   The chimeric oligonucleotide of claim 10 wherein the region which is a substrate for RNAse H is flanked by regions which are modified to enhance target affinity.

12.   The chimeric oligonucleotide of claim 10 wherein the region of the chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

13.   The chimeric oligonucleotide of claim 12 wherein the modification at the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

14.   The chimeric oligonucleotide of claim 10 wherein the region which is a substrate for RNAse H comprises 2'-deoxynucleotides.

15.   The chimeric oligonucleotide of claim 14 wherein the 2'-deoxynucleotide region is four to nine nucleotides long.

16.   The chimeric oligonucleotide of claim 15 wherein the 2'-deoxynucleotide region is five to seven nucleotides long.

17.   The chimeric oligonucleotide of claim 10 wherein at least one of the linking groups between nucleotide units comprises a sulfur-containing modification.

18.   The chimeric oligonucleotide of claim 17 wherein the

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sulfur-containing   modification   is   a   phosphorothioate
modification.

19.   The   chimeric   oligonucleotide   of   claim   10
specifically hybridizable with a translation initiation site or
5   codon 12 of the ras gene.

20.   The chimeric oligonucleotide of claim 10 in a
pharmaceutically acceptable carrier.

21.   The chimeric oligonucleotide of claim 10 having a
sequence selected from the group consisting of SEQ ID NO: 1, 2,
10   3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

22.   A method of modulating the expression of the human
H-ras gene comprising contacting tissues or cells containing
the human H-ras gene with an oligonucleotide comprising from 8
to 30 nucleotide units specifically hybridizable with selected
15   DNA or mRNA deriving from the human H-ras gene, and modulating
the expression of the gene.

23.   The method of claim 22 wherein said oligonucleotide
is specifically hybridizable with a translation initiation site
or codon 12 of the human H-ras gene.

20   24.   The method of claim 22 wherein at least one of the
linking groups between nucleotide units of the oligonucleotide
comprises a sulfur-containing modification.

25.   The method of claim 24 wherein the sulfur-containing
modification is a phosphorothioate modification.

25   26.   The method of claim 22 wherein at least one of the
nucleotide units of the oligonucleotide is modified at the 2'
position.

27.   The method of claim 26 wherein said modification at

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the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

28. The method of claim 22 wherein said oligonucleotide is a substrate for RNAse H and comprises at least one modification to enhance nuclease resistance and at least one modification to enhance affinity for said ras DNA or mRNA.

29. The method of claim 22 wherein said oligonucleotide is a chimeric oligonucleotide which is stabilized against nucleases and which contains a first region comprising at least one nucleotide which is modified to enhance target affinity and a second region which is a substrate for RNAse H.

30. The method of claim 29 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H is flanked by regions which are modified to enhance target affinity.

31. The method of claim 29 wherein the region of the chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

32. The method of claim 31 wherein the modification at the 2' position of the chimeric oligonucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

33. The method of claim 29 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H comprises 2'-deoxynucleotides.

34. The method of claim 33 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is four to nine nucleotides long.

35. The method of claim 34 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is five to seven nucleotides long.

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36.   The method of claim 22 wherein said oligonucleotide
is selected from the group consisting of SEQ ID NO: 1, 2, 3, 4,
5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

37.   A method of detecting the presence of the H-ras gene
5 in cells or tissues comprising contacting cells or tissues with
an oligonucleotide comprising from 8 to 30 nucleotide units
specifically hybridizable with selected DNA or mRNA deriving
from the human H-ras gene and detecting if the H-ras gene is
present.

10   38.   The method of claim 37 wherein said oligonucleotide
is specifically hybridizable with a translation initiation site
or codon 12 of the human H-ras gene.

39.   The method of claim 37 wherein at least one of the
linking groups between nucleotide units of the oligonucleotide
15 comprises a sulfur-containing modification.

40.   The method of claim 39 wherein the sulfur-containing
modification is a phosphorothioate modification.

41.   The method of claim 37 wherein at least one of the
nucleotide units of the oligonucleotide is modified at the 2'
20 position.

42.   The method of claim 41 wherein said modification at
the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

43.   The method of claim 37 wherein said oligonucleotide
is   a   substrate   for   RNAse   H   and   comprises   at   least   one
25 modification to enhance nuclease resistance and at least one
modification to enhance affinity for said ras DNA or mRNA.

44.   The method of claim 37 wherein said oligonucleotide
is   a   chimeric   oligonucleotide   which   is   stabilized   against
nucleases and which contains a first region comprising at least

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one nucleotide which is modified to enhance target affinity and a second region which is a substrate for RNAse H.

45. The method of claim 44 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H is
5 flanked by regions which are modified to enhance target affinity.

46. The method of claim 44 wherein the region of the chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

10   47. The method of claim 46 wherein the modification at the 2' position nucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

48. The method of claim 44 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H
15 comprises 2'-deoxynucleotides.

49. The method of claim 48 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is four to nine nucleotides long.

50. The method of claim 49 wherein the 2'-
20 deoxynucleotide region of the chimeric oligonucleotide is five to seven nucleotides long.

51. The method of claim 37 wherein said oligonucleotide is selected from the group consisting of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

25   52. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human H-ras gene and inhibiting the proliferation of

the cells.

53.   The method of claim 52 wherein said oligonucleotide
is specifically hybridizable with a translation initiation site
or codon 12 of the human H-ras gene.

5   54.   The method of claim 52 wherein at least one of the
linking groups between nucleotide units of the oligonucleotide
comprises a sulfur-containing modification.

55.   The method of claim 54 wherein the sulfur-containing
modification is a phosphorothioate modification.

10   56.   The method of claim 52 wherein at least one of the
nucleotide units of the oligonucleotide is modified at the 2'
position.

57.   The method of claim 56 wherein said modification at
the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

15   58.   The method of claim 52 wherein said oligonucleotide
is selected from the group consisting of SEQ ID NO: 1, 2, 3, 4,
5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

59.   The method of claim 52 wherein said oligonucleotide
is a substrate for RNAse H and comprises at least one
20 modification to enhance nuclease resistance and at least one
modification to enhance affinity for said ras DNA or mRNA.

60.   The method of claim 52 wherein said oligonucleotide
is a chimeric oligonucleotide which is stabilized against
nucleases and which contains a first region comprising at least
25 one nucleotide which is modified to enhance target affinity and
a second region which is a substrate for RNAse H.

61.   The method of claim 60 wherein the region of the
chimeric oligonucleotide which is a substrate for RNAse H is

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flanked by regions which are modified to enhance target affinity.

62.   The method of claim 60 wherein the region of the chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

63.   The method of claim 60 wherein the modification at the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

64.   The method of claim 60 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H comprises 2'-deoxynucleotides.

65.   The method of claim 64 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is four to nine nucleotides long.

66.   The method of claim 65 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is five to seven nucleotides long.

67.   A method of treating conditions arising from the activation of the H-ras oncogene comprising contacting an animal having a condition arising from the activation of H-ras oncogene with a therapeutically effective amount of an oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human H-ras gene.

68.   The method of claim 67 wherein said oligonucleotide is specifically hybridizable with a translation initiation site or codon 12 of the human H-ras gene.

69.   The method of claim 67 wherein at least one of the linking groups between nucleotide units of the oligonucleotide

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comprises a sulfur-containing modification.

70.   The method of claim 69 wherein the sulfur-containing modification is a phosphorothioate modification.

71.   The method of claim 67 wherein at least one of the
5 nucleotide units of the oligonucleotide is modified at the 2' position.

72.   The method of claim 71 wherein said modification at the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

73.   The method of claim 67 wherein said oligonucleotide
10 is selected from the group consisting of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

74.   The method of claim 67 wherein said oligonucleotide is specifically hybridizable with the activated codon 12 of the human H-ras gene.

15   75.   The method of claim 74 wherein said oligonucleotide comprises SEQ ID NO: 3, 4, 5, 6, 11, 12, 13, 14, 15, 16, 17, or 18.

76.   The method of claim 67 wherein said oligonucleotide selectively inhibits the activated human H-ras gene.

20   77.   The method of claim 76 wherein said oligonucleotide is selected from the group consisting of SEQ ID NO: 3, 4, 13, 14, 15, 16 and 17.

78.   The method of claim 67 wherein said oligonucleotide is a substrate for RNAse H and comprises at least one
25 modification to enhance nuclease resistance and at least one modification to enhance affinity for said ras DNA or mRNA.

79.   The method of claim 78 wherein said oligonucleotide

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is a chimeric oligonucleotide which is stabilized against nucleases and which contains a first region comprising at least one nucleotide which is modified to enhance target affinity and a second region which is a substrate for RNAse H.

5   80.   The method of claim 79 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H is flanked by regions which are modified to enhance target affinity.

        81.   The method of claim 79 wherein the region of the
10 chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

        82.   The method of claim 81 wherein the modification at the 2' position of the oligonucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

15   83.   The method of claim 79 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H comprises 2'-deoxynucleotides.

        84.   The method of claim 83 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is four
20 to nine nucleotides long.

        85.   The method of claim 84 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is five to seven nucleotides long.

        86.   A method of detecting activated H-ras based on the
25 differential affinity of particular oligonucleotides for activated H-ras comprising contacting cells or tissues suspected of containing activated H-ras with an oligonucleotide comprising SEQ ID NO: 3, 4, 13, 14, 15, 16, or 17; and contacting an identical sample of cells or tissues with an
30 oligonucleotide comprising SEQ ID NO: 1, 2, 5, 7, 18, or 19.

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87. The method of claim 86 wherein at least one of the linking groups between nucleotide units of at least one of the oligonucleotides comprises a sulfur-containing modification.

88. The method of claim 87 wherein the sulfur-containing
5   modification is a phosphorothioate modification.

89. The method of claim 86 wherein at least one of the nucleotide units of at least one of the oligonucleotides is modified at the 2' position.

90. The method of claim 89 wherein said modification at
10 the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

91. The method of claim 86 wherein at least one of said oligonucleotides is a substrate for RNAse H and comprises at least one modification to enhance nuclease resistance and at least one modification to enhance affinity for activated H-ras
15 DNA or mRNA.

92. The method of claim 86 wherein at least one of said oligonucleotides is a chimeric oligonucleotide which is stabilized against nucleases and which contains a first region comprising at least one nucleotide which is modified to enhance
20 target affinity and a second region which is a substrate for RNAse H.

93. The method of claim 92 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H is flanked by regions which are modified to enhance target
25 affinity.

94. The method of claim 92 wherein the region of the chimeric oligonucleotide which is modified to enhance target affinity comprises nucleotides modified at the 2' position.

95. The method of claim 94 wherein the modification at

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the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

96.   The method of claim 92 wherein the region of the chimeric oligonucleotide which is a substrate for RNAse H comprises 2'-deoxynucleotides.

5   97.   The method of claim 96 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is four to nine nucleotides long.

98.   The method of claim 97 wherein the 2'-deoxynucleotide region of the chimeric oligonucleotide is five
10 to seven nucleotides long.

99.   An antisense oligonucleotide for binding a mutant target mRNA having a sequence which will result in at least one mismatch between the oligonucleotide sequence and the wild type mRNA sequence and a free energy difference for oligonucleotide
15 binding to matched and mismatched RNA of greater than 1 kcal/mol.

100. An antisense oligonucleotide of claim 99 wherein said free energy difference is greater than 2 kcal/mol.

101. An antisense oligonucleotide of claim 100 wherein
20 said free energy difference is about 5 to about 6 kcal/mol.

102. An antisense oligonucleotide for binding a mutant target mRNA having a sequence selected such that there is at least one mismatched base pair between the oligonucleotide and the wild type target mRNA and said mismatch is positioned
25 within the oligonucleotide at a position that hybridizes with a base other than a guanine base of the target mRNA.

103. The oligonucleotide of claim 1 having a sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

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104. A method of modulating the expression of the human H-ras gene comprising contacting tissues or cells containing the human H-ras gene with an oligonucleotide having SEQ ID NO: 2 or SEQ ID NO: 3.

5   105. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an oligonucleotide having SEQ ID NO: 2 or SEQ ID NO: 3.

106. A method of treating conditions arising from the activation of the H-ras oncogene comprising contacting an
10   animal having a condition arising from the activation of the H-ras oncogene with a therapeutically effective amount of an oligonucleotide having SEQ ID NO: 2 or SEQ ID NO: 3.

107. A chimeric oligonucleotide of claim 10 wherein the ras gene is human H-ras.

15   108. A chimeric oligonucleotide of claim 10 wherein the ras gene is human Ki-ras.

109. A chimeric oligonucleotide of claim 108 having a sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28, SEQ
20   ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

110. An oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human Ki-ras gene.

111. The oligonucleotide of claim 110 specifically
25   hybridizable with the 5'-untranslated region, 3'-untranslated region, codon 12 or codon 61 of the human Ki-ras gene.

112. The oligonucleotide of claim 110 wherein at least one of the linking groups between nucleotide units comprises a

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phosphorothioate modification.

113. The oligonucleotide of claim 110 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position.

5   114. The oligonucleotide of claim 113 wherein said modification at the 2' position is a 2'-O-alkyl or a 2'-fluoro modification.

115. The oligonucleotide of claim 110 having a sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO:
10   21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

116. A method of modulating the expression of the human Ki-ras gene comprising contacting tissues or cells containing the human Ki-ras gene with an oligonucleotide comprising from
15   8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA deriving from the human Ki-ras gene, and modulating the expression of the gene.

117. The method of claim 116 wherein the oligonucleotide is specifically hybridizable with the 5'-untranslated region,
20   3'-untranslated region, codon 12 or codon 61 of the human Ki-ras gene.

118. The method of claim 116 wherein at least one of the linking groups between nucleotide units of the oligonucleotide comprises a phosphorothioate modification.

25   119. The method of claim 116 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position.

120. The method of claim 119 wherein said modification at the 2' position of the oligonucleotide is a 2'-O-alkyl or a

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2'-fluoro modification.

121. The method of claim 116 wherein the oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28,
5 SEQ ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

122. A method of detecting the presence of the Ki-ras gene in cells or tissues comprising contacting cells or tissues with an oligonucleotide comprising from 8 to 30 nucleotide units specifically hybridizable with selected DNA or mRNA
10 deriving from the human Ki-ras gene and detecting if the Ki-ras gene is present.

123. The method of claim 122 wherein the oligonucleotide is specifically hybridizable with the 5'-untranslated region, 3'-untranslated region, codon 12 or codon 61 of the human Ki-
15 ras gene.

124. The method of claim 122 wherein at least one of the linking groups between nucleotide units of the oligonucleotide comprises a phosphorothioate modification.

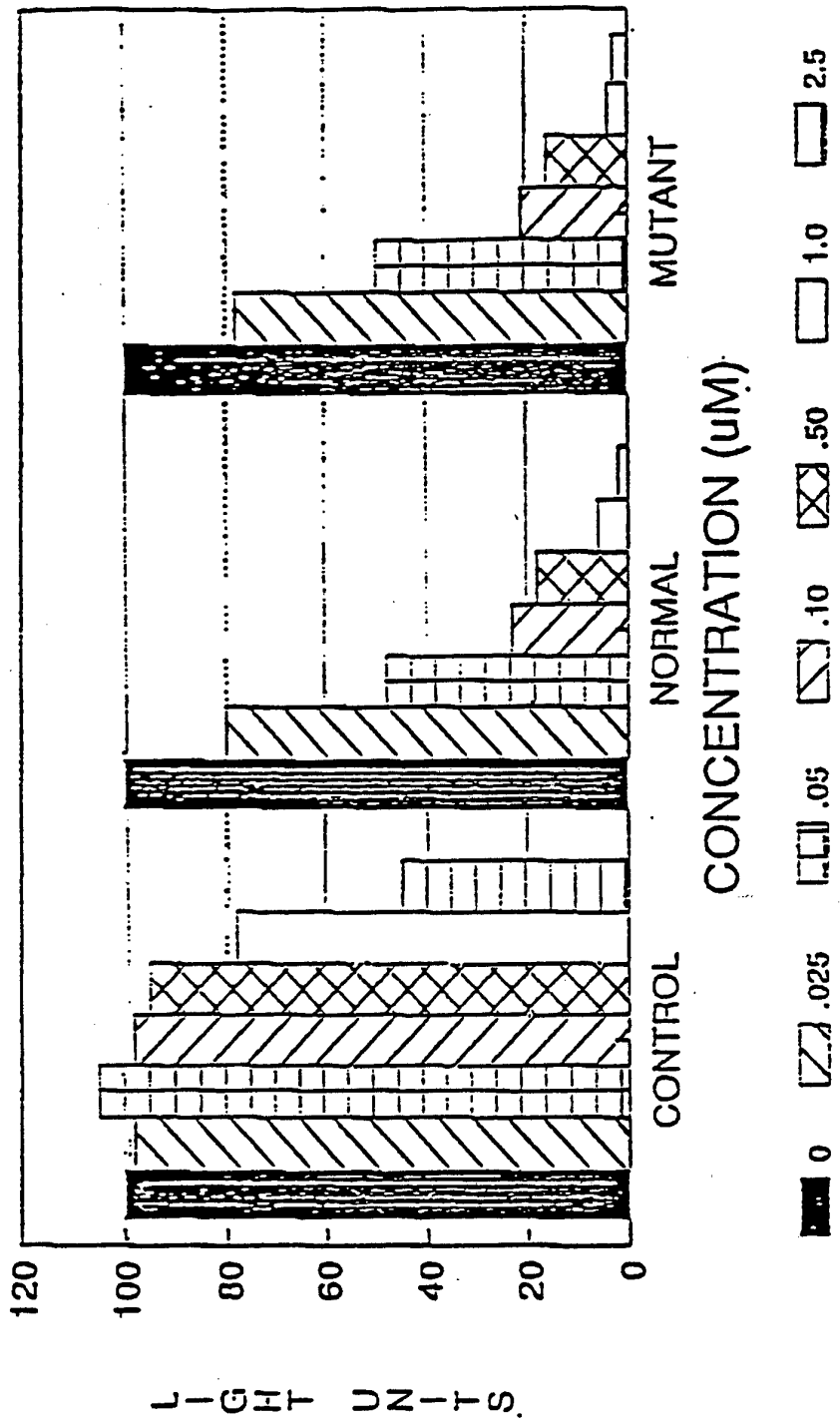125. The method of claim 122 wherein at least one of the
20 nucleotide units of the oligonucleotide is modified at the 2' position.

126. The method of claim 125 wherein said modification at the 2' position of the oligonucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

25   127. The method of claim 122 wherein the oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

128. A method of inhibiting the proliferation of cancer

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cells comprising contacting cancer cells with an
oligonucleotide comprising from 8 to 30 nucleotide units
specifically hybridizable with selected DNA or mRNA deriving
from the human Ki-ras gene and inhibiting the proliferation of
5   the cells.

        129. The method of claim 128 wherein the oligonucleotide
is specifically hybridizable with the 5'-untranslated region,
3'-untranslated region, codon 12 or codon 61 of the human Ki-
ras gene.

10   130. The method of claim 128 wherein at least one of the
linking groups between nucleotide units of the oligonucleotide
comprises a phosphorothioate modification.

        131. The method of claim 128 wherein at least one of the
nucleotide units of the oligonucleotide is modified at the 2'
15   position.
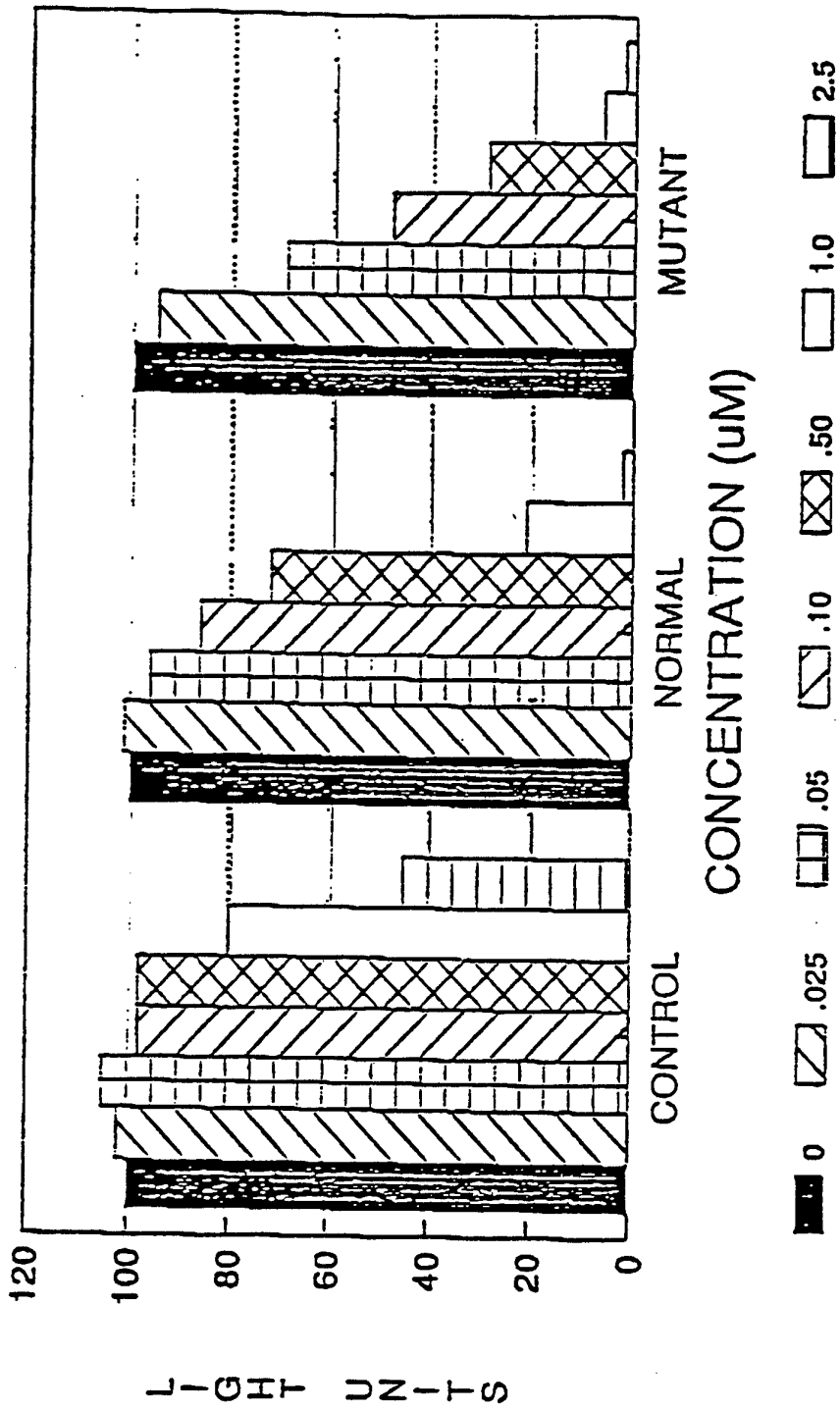

        132. The method of claim 131 wherein said modification
at the 2' position of the oligonucleotide is a 2'-O-alkyl or a
2'-fluoro modification.
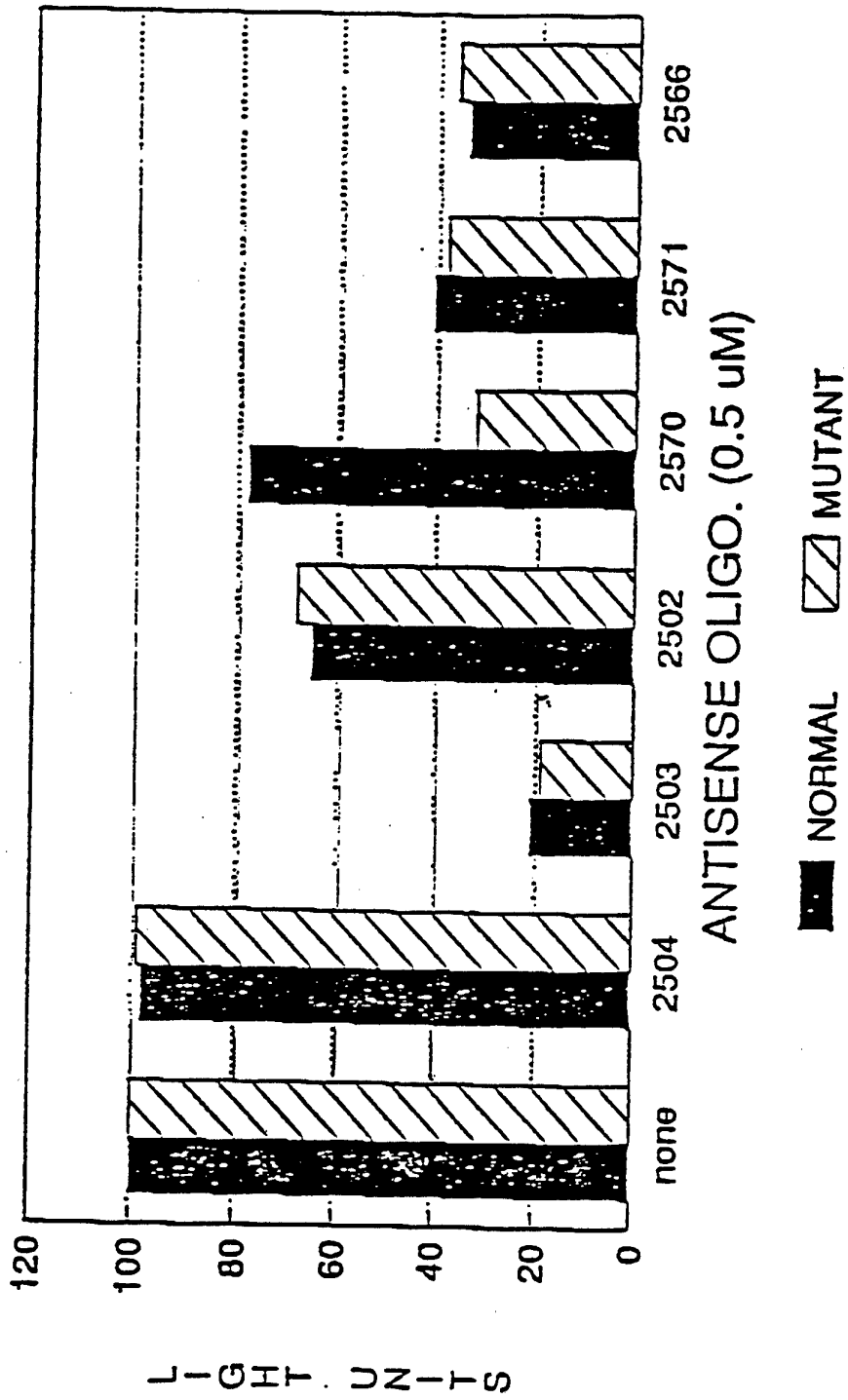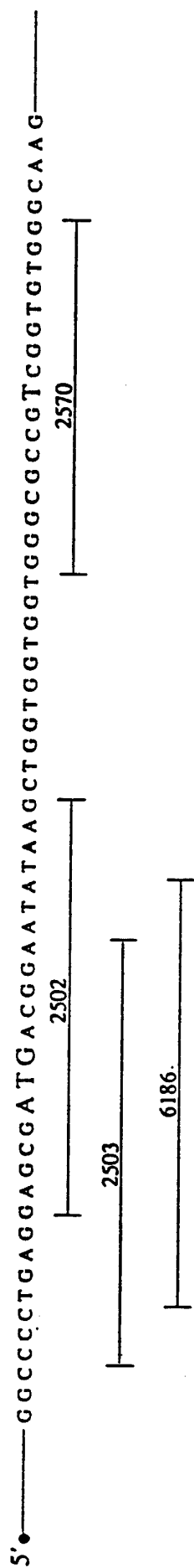

        133. The method of claim 128 wherein the oligonucleotide
20   has a sequence selected from the group consisting of SEQ ID NO:
20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28,
SEQ ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

        134. A method of treating conditions arising from the
activation of the Ki-ras oncogene comprising contacting an
25   animal having a condition arising from hte activation of the
Ki-ras oncogene with a therapeutically effective amount of an
oligonucleotide comprising from 8 to 30 nucleotide units
specifically hybridizable with selected DNA or mRNA deriving
from the human Ki-ras gene.

30   135. The method of claim 134 wherein the oligonucleotide

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is specifically hybridizable with the 5'-untranslated region, 3'-untranslated region, codon 12 or codon 61 of the human Ki-ras gene.

5   136. The method of claim 134 wherein at least one of the linking groups between nucleotide units of the oligonucleotide comprises a phosphorothioate modification.

137. The method of claim 134 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position.

10   138. The method of claim 137 wherein said modification at the 2' position of the oligonucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

139. The method of claim 134 wherein the oligonucleotide has a sequence selected from the group consisting of SEQ ID NO: 15   20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 32 and SEQ ID NO: 33.

140. A method of detecting activated H-ras based on the differential affinity of particular oligonucleotides for activated Ki-ras comprising contacting cells or tissues 20   suspected of containing activated Ki-ras with an oligonucleotide comprising SEQ ID NO: 32 and contacting an identical sample of cells or tissues with an oligonucleotide comprising SEQ ID NO: 20. 21, 22, 26, 28, 31 or 33.

141. The method of claim 140 wherein at least one of the 25   linking groups between nucleotide units of the oligonucleotide comprises a phosphorothioate modification.

142. The method of claim 140 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position.

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143. The method of claim 142 wherein said modification at the 2' position of the oligonucleotide is a 2'-O-alkyl or a 2'-fluoro modification.

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FIGURE 1

FIGURE 2

CONTROL   NORMAL   MUTANT

CONCENTRATION (uM)

LIGHT UNITS

120 100 80 60 40 20 0

■ 0   ▨ .025   ▤ .05   ▧ .10   ▨ .50   ▢ 1.0   ▢ 2.5

FIGURE 3

ANTISENSE OLIGO. (0.5 uM)

■ NORMAL   ⧄ MUTANT

FIGURE 4

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	OLIGO	LENGTH	TARGET
ggccccugaggaccAUGacggaauaaagcuggugguggugggcgccgUcggugugggcaagagugcgcug			
cucgcaactgccuauauuc	2502	20	AUG
gggacuccucgcaactgccu	2503	20	AUG
gcagc	2563	5	CODON 12
ggcagcc	2564	7	CODON 12
cggcagcca	2565	9	CODON 12
gcggcagccac	2567	11	CODON 12
cgcggcagccaca	2568	13	CODON 12
ccgcggcagccacac	2569	15	CODON 12
ccgcggcagccacacc	3426	16	CODON 12
cccgcggcagccacac	3427	16	CODON 12
cccgcggcagccacacc	2570	17	CODON 12
cccgcggcagccacaccc	3428	18	CODON 12
acccgcggcagccacacc	3429	18	CODON 12
acccgcggcagccacaccc	2571	19	CODON 12
cacccgcggcagccacacccg	2566	21	CODON 12
ccacccgcggcagccacacccgt	2560	23	CODON 12
accacccgcggcagccacacccgtt	2561	25	CODON 12
ccccgcggccgccacacc	2907	17	CODON 12 (wild type)

FIGURE 5 A

FIGURE 5 B

2'-O-Me VS DEOXY OLIGONUCLEOTIDES
UNIFORM MODIFICATIONS

FIGURE 6

FIGURE 7

FIGURE 8

A

B

log [oligonucleotide] (M)

C

FIGURE 9
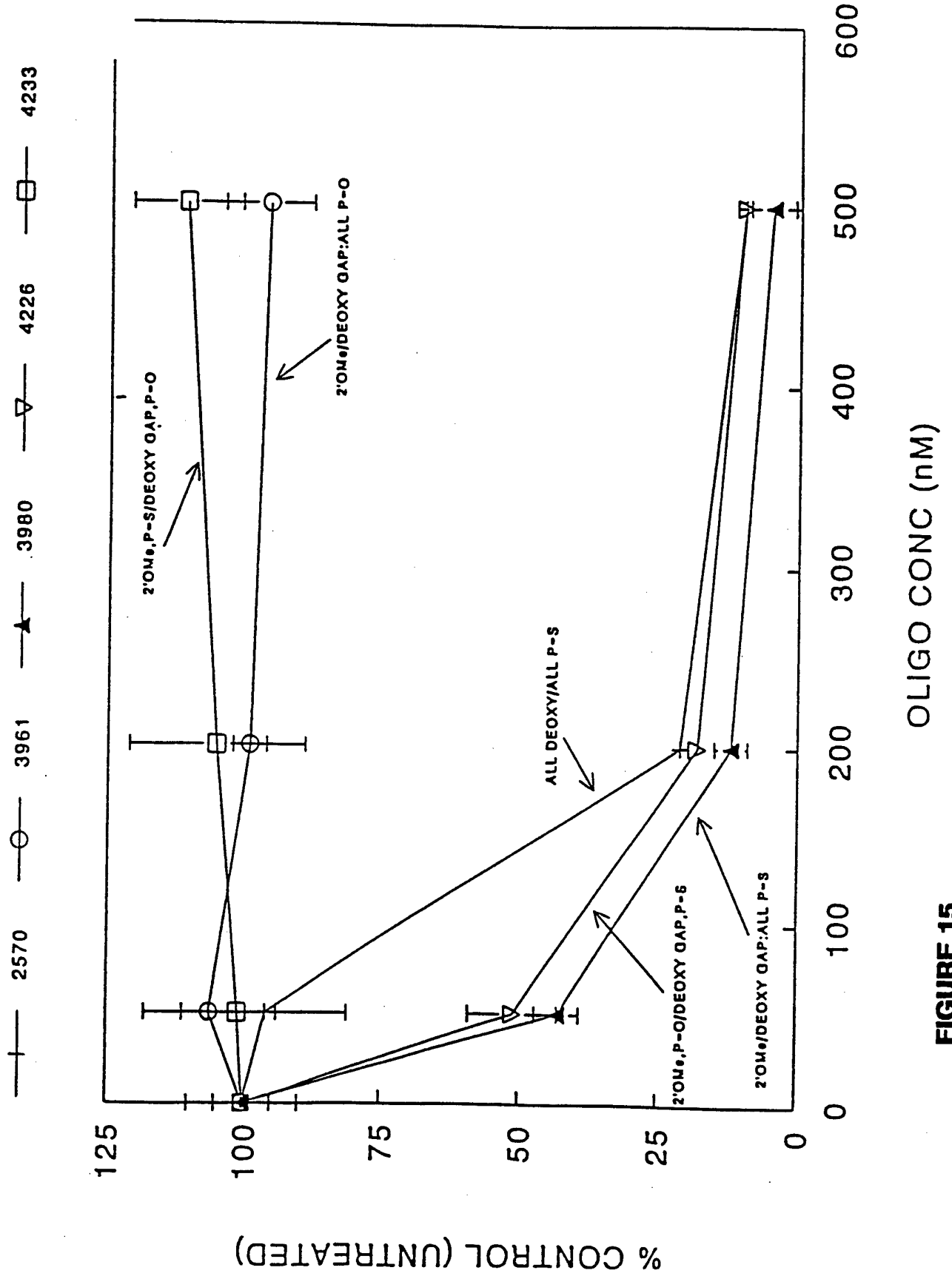
FIGURE 10

FIGURE 11

A.

B.

FIGURE 12

FIGURE 13

FIGURE 14

FIGURE 15

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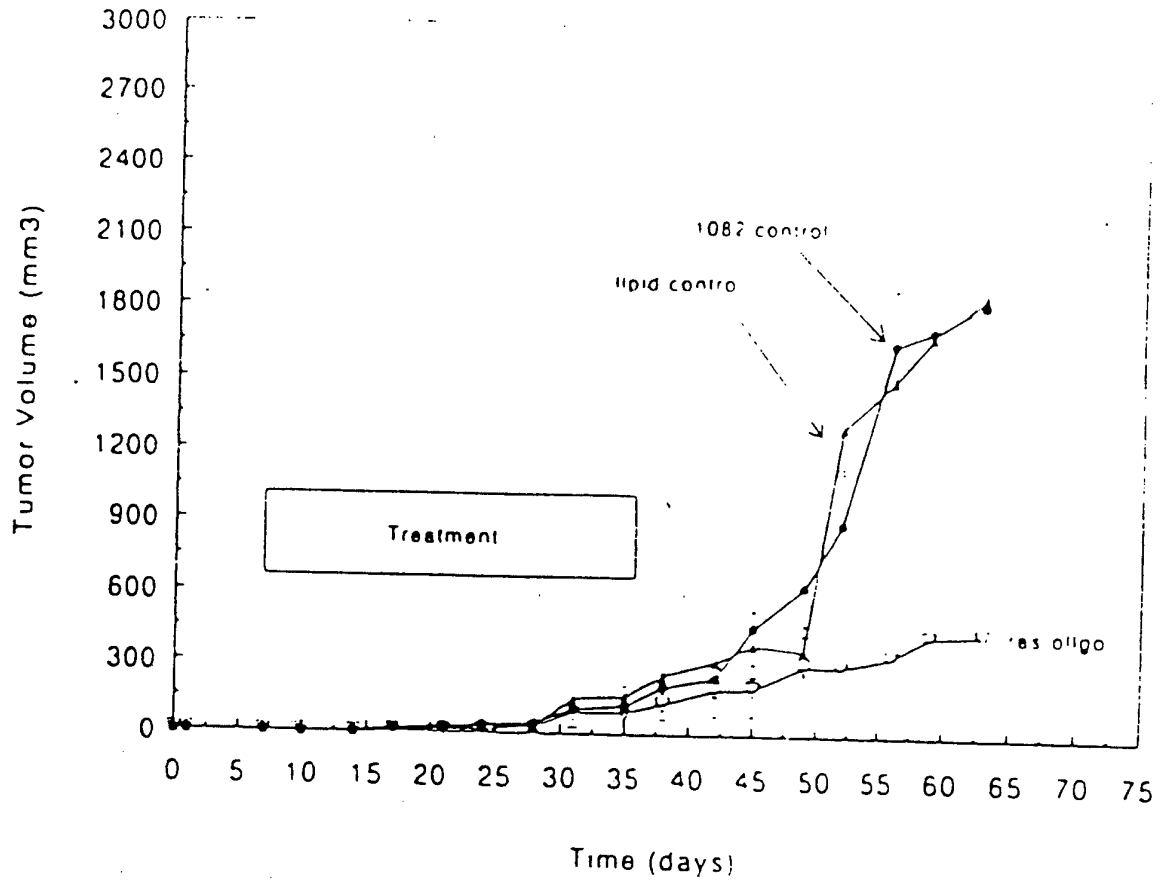

Figure 16

Figure 17

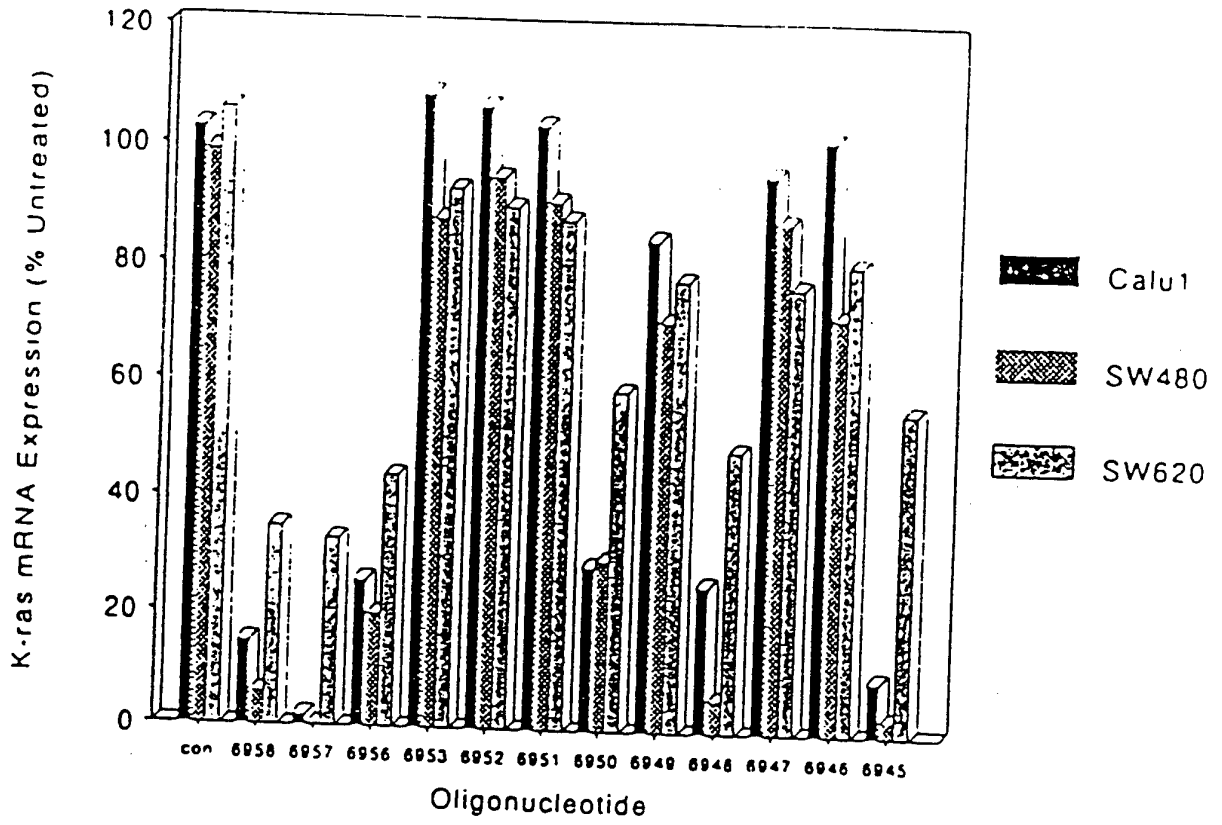Figure 18

Figure 19

Figure 20
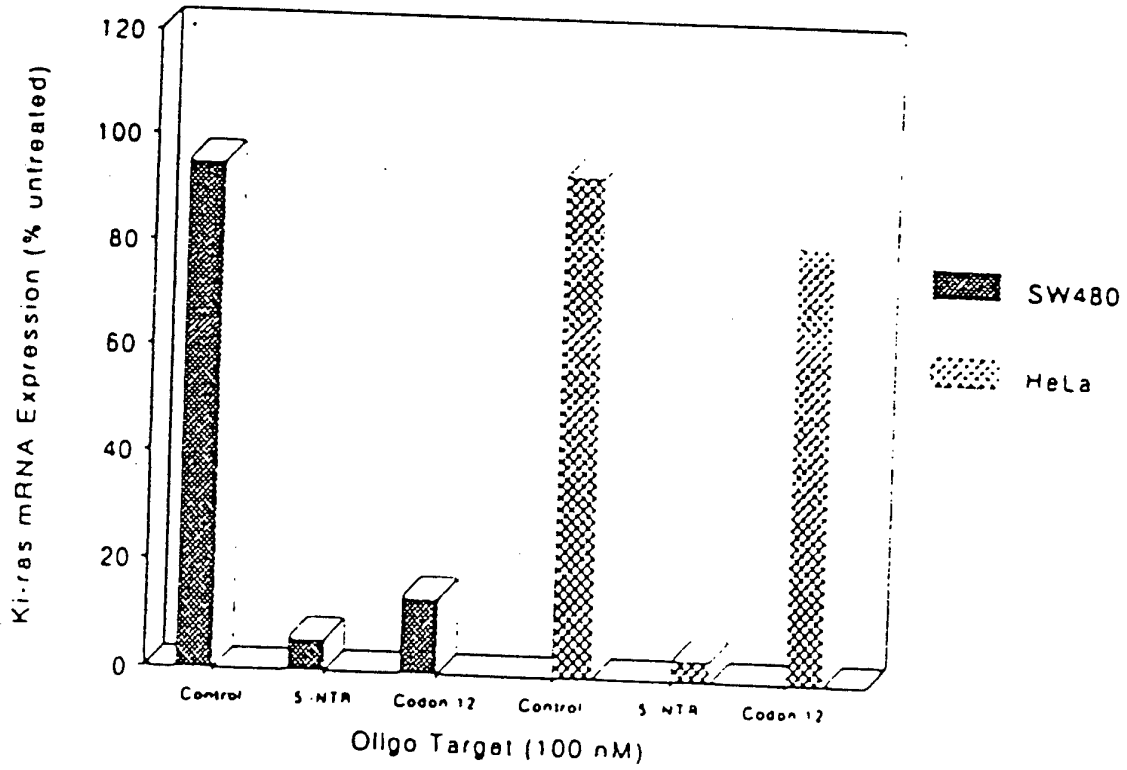
Figure 21

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12N 15/11; C12Q 1/68; A01N 43/04; A61K 31/70

US CL   :536/23.1, 24.1, 24.3; 435/6; 514/44

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   536/23.1, 24.1, 24.3; 435/6; 514/44

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

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

Medline, APS, Dialog, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,871,838 (Bos et al.) 03 October 1989, see entire document.	1-107
Y	Mutation Research, Volume 195, issued 1988, J.L. Bos, "The ras gene family and human carcinogenesis", pages 255-271, see entire document.	1-107
Y	Anti-Cancer Drug Design, Volume 4, issued 1989, E.H. Chang et al., "Comparative inhibition of ras p21 protein synthesis phosphorus-modified antisense oligonucleotides", pages 221-232, see entire document.	1-143

[X]   Further documents are listed in the continuation of Box C.   [ ]   See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 1993	29 DEC 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KATHLEEN L. CHOI
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Oncogene Research, Volume 5, issued 1990, Y. Daaka et al., "Target dependence of antisense oligodeoxynucleotide inhibition of c-Ha-ras p21 expression and focus formation in T24-transformed NIH3T3 cells", pages 267-275, see entire document.	<u>1-2, 10-11, 15-16, 27</u> 3-9, 12-14, 17-26, and 28-107
X Y	The EMBO, Volume 10, No. 5, issued 1991, T. Saison-Behmoaras et al., "Short modified antisense oligonucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation", pages 1111-1118, see entire document.	<u>1-2, 10-11, 15-16, 27</u> 3-9, 12-14, 17-26, and 28-107
Y	Journal of Molecular Biology, Volume 86, issued 1974, P.N. Borer et al., "Stability of ribonucleic acid double-stranded helices", pages 843-853, see entire document.	99-102
Y	Proceedings of National Academy of Science USA, Volume 87, issued February 1990, S. Agrawal et al., "Site-specific excision from RNA by RNase H and mixed-phosphate-backbone oligodeoxynucleotides", pages 1401-1405, see entire document.	6-8, 10-19, 21, 26-36, 41-51, 56-57, 59-66, 71-85, 89-98, 113-115, 120, 125-126, 131-132, and 142-143
Y	EP, A2, 0 260 032 (Ootsuka et al.) 16 March 1988, see entire document.	6-8, 10-19, 21, 26-36, 41-51, 56-57, 59-66, 71-85, 89-98, 113-115, 125-126, 131-132, and 142-143
Y	Nature, Volume 304, issued 11 August 1983, J.P. McGrath et al., "Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene", pages 501-506, see entire document.	108-143
Y	Cancer Research, Volume 51, issued T. Mukhopadhyay et al., "Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA", pages 1744-1748, see entire document.	108-143
Y	WO, A, 92/15680 (Roth et al.) 17 September 1992, see entire document.	108-143
Y	WO, A, 91/12323 (Pederson et al.) 22 August 1991, see entire document.	1-143