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(54) Title: FORMATION OF TRIPLE HELIX COMPLEXES USING A NOVEL MOTIF

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(57) Abstract

Methods of recognizing, detecting and/or inhibiting or altering expression of a target sequence of nucleic acid by forming a triple helix complex using a novel motif. Triple helix complexes may be formed with either single stranded or double stranded target sequences. The stable triple helix complexes have been demonstrated to inhibit translation of an RNA target.
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Formation of Triple Helix Complexes
Using A Novel Motif

Background and Introduction to the Invention
Publications and other reference materials referred to herein are incorporated herein by reference.
The present invention is directed to novel methods of detecting, recognizing and/or inhibiting or altering expression of specific sequences of double stranded or single stranded nucleic acids by formation of triple helix complexes.

The homopyrimidine oligonucleotides were said to recognize extended purine sequences in the major groove of double helical DNA via triple helix formation. Specificity was said to be imparted by Hoogsteen base pairing between the homopyrimidine oligonucleotide and the purine strand of the Watson-Crick duplex. DNA triple helical complexes containing cytosine and thymidine on the third strand have been reported to be stable in slightly acidic to neutral solutions (pH 5.0-6.5), respectively, but have been reported to dissociate on increasing pH. Incorporation of modified bases of T, such as 5-bromo-uracil, and C, such as 5-methylcytosine, into the third strand has been reported to increase stability of the triple helix over a higher pH range. In order for cytosine (C) to participate in the Hoogsteen-type pairing, it was thought that a hydrogen must be available on the N-3 of the pyrimidine ring for
hydrogen bonding. Accordingly, it has been proposed that cytosine be protonated at N-3.

DNA has been reported to exhibit a variety of polymorphic conformations; such conformations may be essential for biological processes. Modulation of signal transduction by sequence-specific protein-DNA binding and molecular interactions such as transcription, translation and replication, are believed to be dependent upon DNA conformation. Wells, R.D., et al., FASEB J. 2:2939-2949 (1988).

The possibility of developing therapeutic agents which bind to critical regions of the genome and selectively inhibit the function, replication and survival of abnormal cells is an exciting concept. See, e.g., Dervan, P., Science 232:464-471 (1988). Various laboratories have pursued the design and development of molecules which interact with DNA in a sequence-specific manner. Such molecules have been proposed to have far-reaching implications for the diagnosis and treatment of diseases involving foreign genetic materials (such as viruses) or alterations to genomic DNA (such as cancer).

Nuclease-resistant nonionic oligodeoxynucleotides (ODN) having a methylphosphonate ("MP") backbone have been studied in vitro and in vivo as potential anticancer, antiviral and antibacterial agents. Miller, P.S., et al. Anti-Cancer Drug Design, 2:117-128 (1987). The 5'-3' linked internucleoside bonds of these analogs are said to approximate the conformation of phosphodiester bonds in nucleic acids. With methylphosphonates, it has been proposed that the phosphate backbone is rendered neutral by methyl substitution of one anionic phosphoryl oxygen, which is thought to decrease inter and intra-strand repulsion due to the charged phosphate groups. Miller, P.S. et al., Anti-Cancer Drug Design 2:117-128 (1987). Oligodeoxynucleoside analogs with a methylphosphonate
backbone are believed to penetrate living cells and have been reported to inhibit mRNA translation in globin synthesis and vesicular stomatitis viral protein synthesis and to inhibit splicing of pre-mRNA in inhibition of herpes simplex virus (HSV) replication. Mechanisms of action for inhibition by the MP analogs include formation of stable complexes with complementary RNA and/or DNA.

Nonionic oligonucleoside alkyl- and aryl-phosphonate analogs complementary to a selected single stranded foreign nucleic acid sequence are reported to be able to selectively inhibit the function or expression of that particular nucleic acid without disturbing the function or expression of other nucleic acids present in the cell, by binding to or interfering with that nucleic acid. (See, e.g., U.S. Patent Nos. 4,469,863 and 4,511,713). The use of complementary nuclease-resistant nonionic oligonucleoside methylphosphonates which are taken up by mammalian cells to inhibit viral protein synthesis in certain contexts, including Herpes simplex virus-1 is described in U.S. Patent No. 4,757,055.

The use of anti-sense oligonucleotides or phosphorothioate analogs complementary to a part of viral RNA to interrupt the transcription and translation of viral mRNA into protein has been proposed. The anti-sense constructs can bind to viral mRNA and were thought to obstruct the cell's ribosomes from moving along the mRNA and thereby halt the translation of mRNA into protein, a process called "translation arrest" or "ribosomal-hybridization arrest." Yarochan, et al., "AIDS Therapies", Scientific American, pages 110-119 (October, 1988).

The inhibition of infection of cells by HTLV-III by administration of oligonucleotides complementary to highly conserved regions of the HTLV-III genome
necessary for HTLV-III replication and/or expression is reported in U.S. Patent No. 4,806,463. The oligonucleotides were said to affect viral replication and/or gene expression as assayed by reverse transcriptase activity (replication) and production of viral proteins p15 and p24 (gene expression).


PCT Published Application WO 91/06626 described oligonucleotides which are said to have tandem sequences of inverted polarity and which are said to be useful for forming an extended triple helix with a double helical nucleotide duplex. The inverted polarity was said to stabilize the single strand oligonucleotides to exonuclease degradation.

Summary of the Invention

The present invention is directed to methods of selectively detecting, recognizing and/or inhibiting or altering expression of a specific target sequence of a nucleic acid by formation of a triple helix complex. According to one aspect, the target sequence is single stranded.

Thus, in one aspect, the present invention is directed to formation of a triple helix complex by binding together a First Strand, a Second Strand and a Third Strand, wherein the target sequence is one of the strands and the other strands are Oligomers which are optionally covalently linked. The triple helix complex is formed by binding the First Strand with Second and Third Strands which are substantially identical in nucleoside sequence to each other and one of which is sufficiently complementary to the First Strand to bind
thereto by Watson-Crick base pairing. According to one alternative of this aspect, the First Strand is the target sequence. Alternatively, the target sequence may be either the Second Strand or the Third Strand.

Preferably the Second and Third Strands bind parallel to each other and anti-parallel to the First Strand.

In another aspect, the present invention is directed to methods of inhibiting or altering expression of a nucleic acid having a single-stranded target sequence by binding together a First Strand, a Second Strand, and a Third Strand to give a triple stranded complex wherein one of the strands is the target sequence and the other strands are Oligomers which are optionally covalently linked. The method comprises contacting the First Strand with Second and Third Strands which have nucleoside sequences substantially identical to each other and one of which is sufficiently complementary to the First Strand to be able to bind thereto by Watson-Crick base pairing. Preferably, the Second and Third Strands bind parallel to each other and anti-parallel to the First Strand.

According to an additional aspect, the present invention is directed to a method of forming a stable triple helix complex by binding together a First Stand, a Second Strand and a Third Strand wherein one of the strands is a target sequence of single stranded RNA and the two other strands are optionally covalently linked Oligomers and wherein the Second and Third Strands have the same strand polarity, have substantially identical nucleoside sequences, and are independently substantially complementary to the target sequence. The RNA target sequence is preferably a mRNA or a pre-mRNA.

In the above methods, the target sequence may be either of the First, Second or Third Strands, but is generally, the First Strand. Preferably the Second and Third Strands bind parallel to each other and anti-
parallel to the First Strand. Preferably the First Stand comprises predominately pyrimidine nucleosides and the Second and Third Strands comprise predominately purine nucleosides.

Alternatively, another aspect of the present invention is directed to a method of inhibiting expression of a nucleic acid having a single-stranded target sequence by binding together a First Strand, a Second Strand and a Third Strand wherein one of the strands is the target sequence and the other strands are Oligomers which are optionally covalently linked. This method comprises contacting the First Strand with Second and Third Strands which are parallel to each other and have substantially identical nucleotide sequences to each other, and wherein the Second Strand is sufficiently complementary to the First Strand to bind thereto by Watson-Crick base pairing and wherein the Third Strand hydrogen bonds with both the Second Strand and the First Strand to give a triple helix complex. Preferably, the target sequence is the First Strand. However, if desired, the target sequence can be either of the Second or the Third Strands. It is especially preferred that the First Strand comprises a predominantly pyrimidine sequence.

According to a preferred aspect, the present invention is directed to methods of forming a triple helix complex using a bridging motif of hydrogen bonding between bases of the nucleosides of the First, Second and Third Strands. According to this bridging motif, the bases of the nucleosides of the Second and Third Strands hydrogen bond with each other and each also hydrogen bonds with the bases of the nucleosides of the First Strand.

Therefore, according to a preferred aspect, methods of detecting or recognizing a nucleic acid having a single stranded target sequence by binding First, Second
and Third Strands wherein one strand is the target sequence and the other strands are Oligomers which are optionally covalently linked are provided. According to this preferred aspect, the Second Strand is sufficiently complementary to the first Strand to bind to the First Strand by Watson-Crick base pairing and the Third Strand hydrogen bonds with and binds to both the First Strand and the Second Strand by recognizing each Watson-Crick base pair. According to one alternative, the target sequence is the First Strand. Alternatively, the target sequence may be either the Second or Third Strand. Preferably, in the triple helix complex, the Second and Third Strands bind parallel to each other and anti-parallel to the First Strand.

We believe that the bridging motif of the present invention may also be used to recognize or detect double stranded target sequences. According to this alternative aspect, a double stranded target sequence which has a sense strand and an anti-sense strand may be detected or recognized by contacting the target sequence with a Third Strand having a nucleoside sequence substantially identical to the nucleoside sequence of one strand (either the sense strand or the anti-sense strand) wherein a base of the Third Strand hydrogen bonds to a corresponding base of each strand of the target sequence to give a triplet and wherein multiple triplets are formed to give a triple helix complex.

According to a preferred aspect, with a single stranded target sequence, Second and Third Strands are added. The Second Strand specifically hydrogen bonds to the target sequence by Watson-Crick base pairing. The bases of the Third Strand specifically hydrogen bond with and, thus, bind to bases of both members of the corresponding Watson-Crick base pairs. The triad formed by the hydrogen bonding of the Third Strand base with
both members of a Watson-Crick base pair is termed a "bridging triad".

According to an especially preferred aspect, we have found that triple helix complexes which comprise a plurality of bridging triads may be formed using a Third Strand which has the same strand polarity and is substantially the same with respect to nucleoside sequence as one of the two strands of a double-stranded target sequence or, in the case of a single-stranded target, has the same strand polarity and is substantially the same with respect to nucleoside sequence as either the Second Strand or the target sequence. According to a particularly preferred aspect, with single stranded target sequences, the Third Strand preferably has the same strand polarity (i.e., is parallel to) and nucleoside sequence as the Second Strand.

The Oligomers used according to the present invention preferably comprise Oligomers which have a neutral backbone. Preferably these Oligomers are substantially neutral. More preferably, neutral Oligomers are used. Particularly preferred are substantially neutral methyl phosphonate Oligomers. According to an especially preferred aspect, neutral methyl phosphonate Oligomers are employed.

Definitions

As used herein, the following terms have the following meanings unless expressly stated to the contrary.

The term "purine" or "purine base" includes not only the naturally occurring adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs modified at the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having
carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

The term "nucleoside" includes a nucleosidyl unit and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their bases, but also analogs and modified forms of the naturally-occurring bases, including the pyrimidine-5-donor/acceptor bases such as pseudouracil and pseudouracil and other modified bases (such as 8-substituted purines). In RNA, the 5-carbon sugar is ribose; in DNA, it is a 2'-deoxyribose. The term nucleoside also includes other analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

The term "phosphonate" refers to the group O=P-R

wherein R is hydrogen or an alkyl or aryl group.

Suitable alkyl or aryl groups include those which do not sterically hinder the phosphonate linkage or interact with each other. The phosphonate group may exist in either an "R" or an "S" configuration. Phosphonate groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

The term "phosphodiester" or "diester" refers to

O=P-O'

wherein phosphodiester groups may be used as internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.
A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the phosphorus backbone has been replaced by other chemical moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

The term "oligonucleoside" or "Oligomer" refers to a chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be obtained by enzymatic means. Thus, the term "Oligomer" refers to a chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs, alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as phosphotriesters and other oligonucleoside analogs and modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/nucleotide polymers wherein one or more of the phosphorus group linkages between monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, or a carbamate linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone of the non-nucleoside
are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "alkyl- or aryl-phosphonate Oligomer" refers to Oligomers having at least one alkyl- or aryl-phosphonate internucleosidyl linkage. Suitable alkyl- or aryl-phosphonate groups include alkyl- or aryl-groups which do not sterically hinder the phosphonate linkage or interact with each other. Preferred alkyl groups include lower alkyl groups having from about 1 to about 6 carbon atoms. Suitable aryl groups have at least one ring having a conjugated pi electron system and include carbocyclic aryl and heterocyclic aryl groups, which may be optionally substituted and preferably having up to about 10 carbon atoms.

The term "methylphosphonate Oligomer" (or "MP-Oligomer") refers to Oligomers having at least one methylphosphonate internucleosidyl linkage.

The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl linkages such as alkyl- or aryl-phosphonate linkages, alkyl- or aryl-phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and non-phosphorus-containing internucleosidyl linkages, such as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleoside or nucleoside/non-nucleoside polymer and a second molecule which comprises a conjugation partner. Such conjugation partners may
comprise intercalators, alkylating agents, binding 
substances for cell surface receptors, lipophilic 
agents, nucleic acid modifying groups including photo-
cross-linking agents such as psoralen and groups capable 
of cleaving a targeted portion of a nucleic acid, and 
the like. Such conjugation partners may further enhance 
the uptake of the Oligomer, modify the interaction of 
the Oligomer with the target sequence, or alter the 
pharmacokinetic distribution of the Oligomer. The 
essential requirement is that the oligonucleoside or 
nucleoside/non-nucleoside polymer that the Oligomer 
conjugate comprises be substantially neutral. 

The term "substantially neutral" in referring to an 
Oligomer refers to those Oligomers in which at least 
about 80 percent of the internucleosidyl linkages 
between the nucleoside monomers are nonionic linkages. 

The term "neutral alkyl- or aryl- phosphonate 
Oligomer" refers to neutral Oligomers having neutral 
internucleosidyl linkages which comprise at least one 
alkyl- or aryl- phosphonate linkage. 

The term "neutral methylphosphonate Oligomer" 
refers to neutral Oligomers having internucleosidyl 
linkages which comprise at least one methylphosphonate 
linkage.

The term "triplet" or "triad" refers a hydrogen 
bonded complex of the bases of three nucleosides between 
a base (if single stranded) or bases (if double 
stranded) of a target sequence, a base of a Second 
Strand and a Third Strand (if a single stranded target 
sequence) or a base of a Third Strand (if a double-
stranded target). Examples of some possible bridging 
triads are depicted on Figure 6.

The term "substantially identical" in referring to 
the nucleoside sequences of Second and Third Oligomers 
denotes a sequence homology of 90% or greater (i.e., the 
oligomers are 90% to 100% homologous).
Brief Description of the Drawings

Figure 1 depicts an autoradiograph of a non-denaturating polyacrylamide gel which demonstrates formation of triple helix complexes between an RNA target sequence and methylphosphonate Second and Third Strands as described in Example 2.

Figure 2 depicts an autoradiograph of a non-denaturating polyacrylamide gel which demonstrates formation of triple helix complexes between an RNA target and methylphosphonate Second and Third Strands as described in Example 1.

Figure 3 depicts an autoradiograph of a non-denaturating polyacrylamide gel which demonstrates formation of triple helix complexes between an RNA target sequence and methylphosphonate Second and Third Strands containing a single diester linkage at their 5'-ends.

Figure 4 depicts thermal denaturation profiles for methylphosphonate Oligomer sequences 2100 (-----), 2101 (------), 2102 (-----), and 2106 (-----) in the presence of their corresponding, perfectly complementary RNA Oligomers.

Figure 5 depicts an autoradiograph of a protein gel which demonstrates sequence specific inhibition of protein synthesis by triple strand forming methylphosphonate Oligomers.

Figure 6 depicts hydrogen bonding schemes for some of the possible bridging triads formed according to the methods of the present invention.

Figure 7 depicts an autoradiograph of a protein gel which depicts sequence specific inhibition of protein synthesis by triple strand forming methylphosphonate oligomers as compared with diester oligomers having the same nucleoside sequence as described in Example 8.

Figure 8 depicts an autoradiograph of a protein gel which depicts sequence specific inhibition of protein
synthesis by triple strand forming methylphosphonate oligomers having complementary to a target sequence compared to a methylphosphonate oligomer having two mismatches as described in Example 9.

Detailed Description of the Invention

General Strategy

The antisense strategy for development of specifically synthesized Oligomers as sequence-specific/gene-specific therapeutic and diagnostic agents is now a major direction for drug development.

There are two general classes of triple-stranded motifs commonly known in the literature as type 1 and type 2. Type 1 is typically defined as a polypyrimidine Third Strand binding to the major groove of a double-stranded DNA, in a parallel orientation with respect to the homopurine strand of the duplex. Recognition is achieved through Hoogsteen hydrogen bonding of thymine with the adenine base in A-T diads and protonated cytosine with the guanosine base in G-C diads. Type 2, on the other hand, is defined as a polypurine Third Strand binding to a homopurine strand in a duplex in an antiparallel orientation. It is generally thought that the purine bases of the Third Strand hydrogen bond specifically to the same bases in the purine strand of the duplex (i.e., A to A and G to G). For this particular motif, a Third Strand is required which contains the same sequence of bases as the Second Strand but in an antiparallel orientation, that is, the Second and Third Strands are distinct sequences in reverse orientation.

Because type 1 and type 2 triple strands bind in reverse orientations with respect to the polypurine strand of the duplex, it was generally thought that one could not mix the two motifs without modifying the polarity of the backbone (i.e., shift from 5'→3' to 3'→5', etc.). Therefore, it has been concluded that
alternating purine-pyrimidine sequences would prevent the formation of triplets because half of the bases in the Third Strand would violate the classical motifs. (See also, for example, Letai, A.G., Palladino, M.A., Fromm, E. Rizzo, V. and Fresco, J.R., "Specificity in Formation of Triple-Stranded Nucleic Acid Helical Complexes: Studies with Agarose-Linked Polynucleotide Affinity Columns", *Biochemistry*, 27:9108-9112 (1988)).

Using neutral backbone analogs we have demonstrated that mixed purine-pyrimidine sequences (alternating GT, GU and CA) can form triple stranded complexes at physiological pH and higher (7.2-8.2). This would be unexpected based on classical arguments as described above. In addition, classical binding motifs having cytosine in the Third Strand require it to be protonated; accordingly, one would not expect stable triple stranded complexes at pH values greater than about 7.0. Furthermore, our experiments with Oligomers having random sequences of predominantly purine nucleosides (including sequences containing up to 2 pyrimidines in a 16 mer) show clear evidence for 2:1 triple stranded complexes with a predominantly pyrimidine single-stranded target. Such complexes would not be predicted if the two predominately purine Oligomer strands were binding antiparallel to each other, and, thus, the Oligomers must be binding in a parallel orientation, which violates the classical pur-pur-pyr motif (type 2).

We believe that the fact that the methylphosphonate backbone is uncharged probably contributes to the binding energy (and thus stability) of these "non-classical" triplets. We have generated computer models of a "bridging" parallel motif which are consistent with and may explain our data. In this motif, the backbone of the Third Strand is closer to that of the pyrimidine-rich strand in the duplex compared to distances observed
for the classical motifs (type 1 and type 2). If the two predominately purine strands of the triple helix were negatively charged, one would expect more charge-repulsion than in a "bridging" motif, compared to the case where one or both of the these strands were neutral, as in the case of methylphosphonates.

Thus, our experiments are consistent with and support our proposed "bridging" motif, where the two predominantly purine (Second and Third) Strands are binding in parallel. The fact that our experiments were done with neutral Oligomers may have contributed to the stability observed in this study; which is consistent with computer modelling as described above.

 Interruption of translation of a target sequence of a mRNA by binding a complementary Oligomer to the target sequence to give a duplex has been studied. In particular, the portion of the mRNA sequence near to the initiation codon has been proposed as a preferred target. Translation interruption experiments using duplexes formed between such mRNA targets and antisense DNA Oligomers have indicated that these complexes are not sufficiently stable to block translation. Even DNA oligomers which form duplexes having high Tm's tend to be stripped away in the absence of ribonuclease H, apparently by ribosomes.

We have found that formation of triple helix complexes of the present invention inhibit ribosomal translation of mRNA.

The method of detecting or recognizing a nucleic acid target sequence using the motif of the present invention allows formation of a triple helix complex with a nucleic acid target sequence which is single stranded or in some circumstances double stranded.

Formation of triple helix complexes according to the motifs of the present invention is particularly advantageous with single-stranded target sequences.
According to a preferred aspect, with the motif of the present invention and when the First Strand is the target sequence, the Second Strand is substantially complementary to the target sequence so as to be capable of forming a Watson-Crick duplex and the Third Strand has substantially the same nucleoside sequence and orientation as the Second Strand. The triple helix complexes formed according to this motif form a compact hydrophobic core when compared to the previously reported Hoogsteen triplet motifs. The triple helix complexes formed according to the motif of the present invention would have the internucleoside linking groups of the target and Third Strands close to each other such that Third Strands having neutral backbones would be advantageous in promoting stability and enhancing binding affinity.

With respect to single stranded target sequences, we have found that two strands of a neutral methylphosphonate Oligomer (Second and Third Strands) and one strand of a complementary synthetic RNA Oligomer (First Strand) form a triple helix complex. According to our experiments, the two methylphosphonate strands bind in a parallel orientation. Experiments involving triple helix formation with methylphosphonate Oligomers of random sequence of A and G nucleosides which would not make triple helix complexes according to any of the "classical" triplet motifs are further evidence for the formation of triads according to the motif of the present invention.

These triple helix complexes formed by binding a target single stranded RNA and two methylphosphonate Oligomers show high affinity (Tm > 50°C). Formation of these triple helix complexes has been shown to dramatically inhibit translation at sub-micromolar concentrations.
Sequence Restriction in Triple Helix Formation

Reported strategies for triple helix formation in an antisense context propose that the Third Strand base reads one base of a duplex.

These reported strategies involved in triple helix formation at specified target sites and, thus, the ability to have a workable therapeutic antisense application through triple helix formation has been limited by the general requirement of a homopurine or homopyrimidine target sequence. In order to be able to read a target having a mixture of purine and pyrimidine bases, means such as switching the strand being read or reversals in Third Strand polarity were proposed.

The methods of the present invention allow formation of a triple helix complex with a target sequence having any sequence of pyrimidine and purine bases.

The triple helix complexes according to the present invention can be formed using Oligomers containing naturally occurring bases (i.e., A, C, G, T or U). Alternatively, if desired for increased stability, certain stabilizing bases such as 2-amino A (for A) or 5-methyl C may be used in place of the corresponding naturally occurring base. These bases may increase stability of the triple helix complex by having increased hydrogen bonding interactions and stacking interactions with other bases. Increased stability may result in increased affinity constants which increase potency.

Oligomer Strands

The two strands used in addition to the strand which is the target sequence may comprise separate Oligomers ("Oligomer Strands") or, if desired, be covalently linked. It is believed that under certain circumstances covalent linking of Oligomers may enhance
blocking of ribosomal translation. According to a preferred aspect, for use with certain target sequences to provide improved stability of binding to a messenger RNA target sequence, the Oligomer Strands may be covalently linked to each other.

Preferably the Oligomer Strands each comprise from about 4 to about 40 nucleosides, more preferably, from about 6 to 30 nucleosides. Especially preferred are Oligomer Strands of about 8 to about 20 nucleosides.

Oligomer Strands having the selected internucleoside linkages may be conveniently prepared according to synthetic techniques known to those skilled in the art. For example, commercial machines, reagents and protocols are available for the synthesis of Oligomers having phosphodiester and certain other phosphorus-containing internucleoside linkages. See also Gait, M.J., Oligonucleotide Synthesis: A Practical Approach (IRL Press, 1984); Cohen, Jack S., Oligodeoxynucleotides Antisense Inhibitors of Gene Expression, (CRC Press, Boca Raton, FL, 1989); and Oligonucleotides and Analogues: A Practical Approach, (F. Eckstein, ed., 1991). Preparation of Oligomers having certain non-phosphorus-containing internucleoside linkages is described in United States Patent No. 5,142,047, the disclosure of which is incorporated herein by reference.

Strand Complementarity

Preferred are Second and Third Strands that each have a corresponding nucleoside complementary to each nucleoside of the First Strand (i.e., have "exact complementarity"). However, included within the scope of the present invention are Second and Third Strands which may lack a complement for each nucleoside in the First Strand, provided that the Second Strand has such binding affinity for the target sequence and the Third
Strand has sufficient binding affinity for the Second Strand that together the Second and Third Strands bind with the First Strand to form a stable triple helix complex and thereby recognize the target sequence or inhibit its expression. Such strands are referred to as being "substantially complementary" or having "substantial complementarity".

The Second Strand and Third Strand each should be independently substantially complementary to the First Strand. The strands are so selected such that there is sufficient hybridization and hydrogen-bonding between the strands for inhibition of expression of the target sequence, and if the target sequence is a portion of a mRNA, inhibition of translation, to occur. Sufficient hybridization and hydrogen-bonding is related to the strength of the hydrogen-bonding between bases as well as the specificity of the complementary strand. The strength of the hydrogen-bonding is influenced by the number and percentage of bases in a strand that are base paired to complementary bases, according to Watson-Crick base pairing. To be specific, the complementary bases of the strand must be sufficient in number so as to avoid non-specific binding to other sequences within a genome and while at the same time small enough in number to avoid non-specific binding between other sequences within a genome and portions of a long strand.

It will also be appreciated that the base sequence of either the Second or the Third Strand need not be 100 percent complementary to the sequence of the First Strand. Preferably the sequence is at least about 80 percent complementary, more preferably at least about 90 percent and even more preferably about 95 percent or more. The Oligomer Strands may optionally include one or more non-nucleoside monomeric units. Such non-nucleoside monomeric units include those described in co-pending U.S. Serial No. 07/565,307, filed August 9,
1990 (also published PCT Application No. WO 92/02532), the disclosure of which is incorporated herein by reference. The strand in question need only be capable of sufficient hybridization or bonding to the other strands to prevent or interfere with expression of the target sequence, such as by preventing normal translation of the target sequence or to specifically recognize the target sequence. Prevention of normal translation of the target sequence occurs when an expression product of the target sequence is produced in an amount significantly lower than would be the result in the absence of the added Oligomer Strands. The expression product is a protein. Measurement of the decrease in production of proteins is well known to those skilled in the art and such methods include quantification by chromatography, biological assay or immunological reactivity.

**Preferred Target Sequences and Second and Third Strands**

According to a preferred aspect of the present invention, methods of inhibiting or altering expression of a single stranded target sequence by formation of a triple helix complex wherein the First Strand comprises a predominately pyrimidine First Strand are provided. By predominately pyrimidine, is meant a nucleoside sequence which comprises at least about 80% pyrimidine nucleosides. In such case, the Second and Third Strands comprise predominantly purine (greater than about 80% purine nucleosides) strands. According to a more preferred aspect the target sequence is the First Strand. Triple helix complexes are formed by contacting a First Strand with Second and Third Strands preferably of the same strand polarity and nucleoside sequence. Preferably, the First Strand is the target sequence and the Second and Third Strands are Oligomers, preferably
substantially neutral Oligomers, more preferably substantially neutral methyl phosphonate Oligomers.

According to an especially preferred aspect, the target sequence is the First Strand which is comprises at least about 85% pyrimidine nucleosides, even more preferably, it comprises an all pyrimidine sequence. By contacting such an all pyrimidine target sequence with a all purine complementary methyl phosphonate Oligomer, (preferably in a ratio of 1:2 target:Oligomer or greater) triple helix complexes having relatively high Tm's (about 45°C or greater at 1 µM Oligomer concentration) are formed. These triple helixes have been demonstrated to exhibit biological inhibition of mRNA target sequences.

Where the target sequence is the First Strand, especially preferred target sequences include predominantly pyrimidine sequences adjacent to the AUG codon. We have found that triple helix complexes formed using complementary substantially neutral Oligomers are more effective in inhibiting biological processes (e.g., translation) than would be predicted by their Tm's alone. With similar targets, DNA Oligomers which form high Tm complexes are less active in inhibiting translation and appear to be stripped away by the ribosomes.

Single negatively charged backbone Oligomers (such as DNA Oligomers) have been reported to have only moderate effects (about 20 to 80 percent inhibition) on the inhibition of translation in cell-free assays, unless an RNaseH activity is present that cleaves the mRNA. (Maher, L.J. and Dolnick, B.J., Nucleic Acids Res. 16:3341-3355 (1988)). The only dramatic inhibitions previously reported at micromolar concentrations have been with long pieces of antisense RNA or DNA (>50 base pairs) or tandem Oligomers. (See, Melton, D.A., Proc. Natl. Acad. Sci. (USA) 82:144-148
(1985); Leibhaber, et al., J. Mol. Biol. 226:001-013 (1992); Maher, L.J. and Dolnick, B.J., Nucleic Acids Res. 16:3341-3355 (1988)). One explanation for the inability of short charged Oligomers to block translation is the existence of a duplex unwinding activity associated with the ribosome (translation machinery). A highly stable complex of a target RNA with a modified Oligomer that is not recognized by the ribosomal unwinding activity might be more effective in inhibiting translation by steric blocking. However, many Oligomers having modified backbones bind to RNA with low affinity. Indeed, we have found that duplexes formed between RNA targets and methylphosphonate Oligomers tend to have lower Tm's than those formed with DNA targets.

However, we have demonstrated that triple helix complexes formed with a RNA target and two methylphosphonates can form and bind with high affinity (Tm > 50°C). These RNA-MP(1:2) triple helix complexes are observed to be more stable than corresponding DNA-MP(1:2) triple helix complexes. This observation is especially surprising in view of the observation that many DNA:MP duplexes are substantially more stable than the corresponding RNA:MP duplex. According to one aspect of the present invention, we have demonstrated that triple helix complexes of a single stranded RNA target sequence and Second and Third Strands which comprise substantially identical methylphosphonate neutral modified Oligomers can dramatically inhibit translation of the RNA target at sub-micromolar concentrations. The high stability of these triple helix complexes along with their novel bridging structure make these complexes more effective in terms of the extent of inhibition and the dose at which inhibition occurs than previously reported [duplex] complexes. (See, e.g., Boizieu, C., Nucleic Acids Res.
12:1113-1119 (1991); and Maher and Dolnick, supra). We believe that the triple helix complexes of the present invention which are resistant to cellular unwinding activity, can either on their own or with the addition of cleaving or cross-linking moieties prove useful in the inhibition of nucleic acid sequences that cause disease or other conditions wherein inhibition or alteration of expression of a particular nucleic acid target sequence would be desirable.

10 Utility and Administration

According to one aspect of the present invention, a specific segment of single stranded nucleic acid may be detected or recognized by binding First, Second and Third Strands to form a triple helix with the single stranded target sequence of a nucleic acid which comprises one strand according to the triple helix forming guidelines described herein. Oligomer Strands may be optionally covalently linked. Detectably labeled Oligomers may be used as proved for use in hybridization assays, for example, to detect the presence of a particular single-stranded nucleic acid sequence.

The present invention also provides a method of preventing or altering expression or function of a selected target sequence of single stranded nucleic acid by binding together First, Second and Third Strands which form a triple stranded helix structure wherein the single stranded target is one of the strands as described above. Formation of the triple stranded helix may prevent expression and/or function by modes such as preventing transcription, preventing binding of effector molecules (such as proteins), etc.

The Second and Third Strands are preferably identical in both strand polarity (when bound in the triple helix complex) and nucleoside sequence.
According to the methods of the present invention, a high affinity complex is formed with a high degree of selectivity. Derivatized Oligomer Strands may be used to detect or locate and then irreversibly modify the target site in the nucleic acid by cross-linking (psoralens) or cleaving one or both strands (EDTA). By careful selection of a target site for cleavage, one of the strands may be used as a molecular scissors to specifically cleave a selected nucleic acid sequence. The Oligomer Strands may be derivatized to incorporate a nucleic acid reacting or modifying group which can be caused to react with the nucleic acid segment or a target sequence thereof to irreversibly modify, degrade or destroy the nucleic acid and thus irreversibly inhibit its functions.

These Oligomer Strands may be used to inactivate or inhibit or alter expression of a particular gene or target sequence of the same in a living cell, allowing selective inactivation or inhibition or alteration of expression. The target sequence may be DNA or RNA, such as a pre-mRNA, an mRNA or an RNA sequence such as an initiator codon, a polyadenylation region, an mRNA cap site or a splice junction. These strands could then be used to permanently inactivate, turn off or destroy genes which produced defective or undesired products or if activated caused undesirable effects.

Another aspect of the present invention is directed to a kit for detecting a particular single stranded nucleic acid sequence which comprises Second and Third Strands at least one of which is detectably labeled and selected to be sufficiently complementary to the target sequence of the single stranded nucleic acid to form a triple helix structure therewith.

Since the Oligomer Strands for use with the methods of the present invention form triple helix complexes or other forms of stable association with transcribed
regions, these complexes are useful in "antisense" therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding Oligomers to inactivate undesirable DNA or RNA sequences in vitro or in vivo.

According to an alternate aspect of the present invention, the target sequence may be double stranded. In such case no Second Strand is used. A complementary Third Strand is added which preferably has the same polarity and is substantially identical in nucleoside sequences to one of the strands of the double stranded target.

Many diseases and other conditions are characterized by the presence of undesired DNA or RNA, which may be in certain instances single stranded form and in other instances in double stranded form. These diseases and conditions can be treated using the principles of antisense therapy as is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA target sequence through complementarity or through any other specific binding means, in the case of the present invention by formation of triple helix complexes according to the binding motifs described herein.

The Oligomers for use in the instant invention may be administered singly, or combinations of Oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

In therapeutic applications, the Oligomers can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. The Oligomer active ingredient is generally combined with a carrier such as
a diluent or excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, or lubricants, depending on the nature of the mode of administration and dosage forms.

Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

For systemic administration, injection may be preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the Oligomers for use with the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank’s solution or Ringer’s solution. In addition, the Oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories. For oral administration, the Oligomers are formulated into conventional as well as delayed release oral administration forms such as capsules, tablets, and tonics.

For topical administration, the Oligomers for use in the invention are formulated into ointments, salves,
eye drops, gels, or creams, as is generally known in the art.

In addition to use in therapy, the methods of the present invention may be used diagnostically to detect the presence or absence of the target DNA or RNA sequences to which the Oligomers specifically bind. Such diagnostic tests are conducted by hybridization through triple helix complex formation which is then detected by conventional means. For example, Oligomers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support detected. Alternatively, the presence of a triple helix may be detected by antibodies which specifically recognize such structures. Means for conducting assays using such Oligomers as probes are generally known.

To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed in specifically limiting the invention and such variations of the invention, now known or later developed, which would within the purview of one skilled in the art are considered to fall within the scope of the present invention as hereinafter claimed.

Examples
Example 1
Triple Helix Complex Formation With Two All-Purine Methylphosphonate Oligonucleosides and One Complementary All-Pyrimidine Ribooligonucleosides

The following three sets of all-purine methylphosphonate oligonucleosides ("MP oligomers") and complementary ribooligonucleosides ("RNA oligomers")
were examined for their ability to form triple helix complexes:

Set #1:  
G2100 5'-AGA-GAG-AGA-GAG-AGA-G-3'  (methylphosphonate)  
R39  3'-UCU-CUC-UCU-CUC-UCAU-C'-5'  (ribooligonucleotide)

5  
Set #2:  
G2101 5'-AGA-AAG-GGA-GAG-GGA-A-3'  (methylphosphonate)  
R289  3'-UCU-UUG-CCU-CCU-CCU-U-5'  (ribooligonucleotide)

Set #2:  
G2106 5'-GAG-GGA-AAG-AGA-AAG-G-3'  (methylphosphonate)  
R84  3'-CUC-CCU-UUC-UUC-UUC-C-5'  (ribooligonucleotide)

Molar extinction coefficients, \( \epsilon_{254} \), for the RNA oligomers in 10 mM sodium phosphate, 10⁻⁵ M EDTA, pH 7 (PE7) were determined by enzymatic digestion with snake venom phosphodiesterase I (P-L Biochemicals, Milwaukee, WI). Typically, 0.2 - 0.3 OD\(_{254} \) unit of RNA oligomer was reacted with 0.2 units of phosphodiesterase in 50 µL of buffer (10 mM Tris-HCl, 2 mM MgCl₂, pH 8.2 for two hours. Blanks were run in the same way without RNA present. The reactions were then taken to dryness by rotary evaporation under vacuum (Speed-Vac™, Savant, Inc.) and resuspended in PE7 buffer. Absorbance readings at 254 nm were then measured for the digested RNA samples and corrected by subtracting the readings from the blanks. Typically, absorbance from triplicate digested and undigested RNA oligomer samples were averaged to give \( A_{254 \ digest} \) and \( A_{254 \ oligomer} \) respectively. The concentration of residues in the digest, \( C_{\text{digest}} \), was calculated from the known sequence and the following mononucleotide extinction coefficients (\( \epsilon_{254} \), M⁻¹ cm⁻¹) at pH 7: \( rA, 13,600; rc, 13,400; rC, 6300 \) and \( ru, 8650 \). The concentration of oligomer, \( C_{\text{oligomer}} \), was then calculated as \( C_{\text{digest}} / (\text{number of residues}) \), and the molar extinction coefficient, \( \epsilon_{254} \), was determined from the relationship \( \epsilon_{254} = A_{254 \ oligomer} / C_{\text{oligomer}} \), where \( A_{254 \ oligomer} \) was determined in 1 cm pathlength cuvettes. Calculated \( \epsilon_{254} \) values for the RNA described in this example were as follows: R39, 11.12 X 10⁴; R289, 11.12 X 10⁴; R84, 11.15 X 10⁴.
Molar extinction coefficients, $\epsilon_{254}$, for the MP oligomers in PE7 buffer containing 25% acetonitrile were determined in a similar manner to that described for the RNA oligomers, except for the following modifications. Chemical digestion was performed by reaction in a solution of 1:9 piperidine:water for four hours at 37°C. Following digestion, the solutions were evaporated to dryness as described above, and then coevaporated with three additions of 100 µL 25% acetonitrile in water.

$A_{254}$ _digest_ values were corrected for the absorbance determined in mock digests without MP oligomer. $C_{\text{digest}}$, $C_{\text{oligomer}}$, and $\epsilon_{254}$ were determined as described above except that the following molar extinction coefficients were used for the mononucleotides: A, 13,600; G, 13,000; C, 6300; T, 6800. Calculated $\epsilon_{254}$ values for the MP oligomers described in this example were as follows: G2100, 16.61 $\times$ 10$^4$; G2101, 18.45 $\times$ 10$^4$; G2106, 17.24 $\times$ 10$^4$.

(a) **UV mixing curve analysis**: The stoichiometries of complex formation between the MP oligomers and their complementary RNA oligomers were determined by comparing the absorbance of equimolar strand concentrations at 270 nm as a function of the mole fraction of MP oligomer present in each sample, $X_{\text{MP}} = C_{\text{MP}}/(C_{\text{MP}} + C_{\text{RNA}})$. Solutions of MP oligomer and complementary RNA oligomer were prepared in 10 mM potassium phosphate, 0.03% potassium sarkosylate, 0.1 mM EDTA, pH 7.2, at different values of $X_{\text{MP}}$ ranging from 0 to 1.0. Each solution had a total strand concentration of 2.4 µM. The resulting solutions were annealed by heating to 80°C and then cooling to 4°C over a period of about four hours. $A_{270}$ values were then determined at 15°C using either a Perkin-Elmer Lambda 3 spectrophotometer or a Cary Model 3E spectrophotometer. Each spectrophotometer was equipped with a variable temperature control device interfaced to an IBM compatible personal computer. Plots of $A_{270}$ versus $X_{\text{MP}}$ at
15°C were biphasic for Set #1 and Set #2, with a single transition point at $X_{MP} = 0.67$. This indicated that MP oligomers G2100 and G2101 primarily formed a triple-stranded complex with their complementary RNA oligomers which was 2:1 MP/RNA. The plot of $A_{270}$ versus $X_{MP}$ at 15°C for Set #3 was triphasic, with two transition points at $X_{MP} = 0.33$ and 0.67. Therefore, MP oligomer G2106 appeared capable of forming two different triple-stranded complexes with its complementary RNA oligomer which were either 2:1 RNA/MP or 2:1 MP/RNA.

(b) Gel-shift analysis: Further evidence for triple-strand formation with MP oligomer G2100 and its complementary RNA oligomer R39 was obtained by polyacrylamide gel electrophoresis. Solutions containing 1:1, 2:1 and 3:1 molar ratios of MP:RNA were prepared in 1.5 mL polypropylene microcentrifuge tubes containing 10 mM potassium phosphate, 0.03% potassium sarkosylate, 0.1 mM EDTA, pH 7.2 and 50,000 cpm of $^{32}$P-labelled RNA. Concentrations of RNA oligomer in each tube were either $10^{-7}$ M, $10^{-6}$ M, or $10^{-5}$ M, total volume = 20 µL. The tubes were heated to 80°C and then cooled to 4°C over a period of about four hours. Next, 5 µL aliquots were removed and diluted with 5 µL of 50% glycerol, 1 X TBE buffer (90 mM Tris-borate, 25 mM disodium EDTA, pH 8.2), 0.1% bromphenol blue on ice. For gel-electrophoresis, a 15% polyacrylamide (5% bisacrylamide) gel (0.5 mm thick X 20 cm wide X 30 cm long) containing 1 X TBE buffer, was submerged in a submarine gel apparatus (Hoeffer Scientific, Inc., San Francisco, CA, Model SE620) containing 1X TBE buffer in the lower reservoir. The buffer in the lower reservoir was cooled to 6°C and the samples were then carefully loaded onto the cooled gel. Electrophoresis was conducted at 500 volts (150 mA) for 90 minutes; the gel temperature was maintained at 6°C. Next, the gel was removed and dried onto a sheet of 3MM blotting paper
using a Bio-Rad Model 583 gel dryer (Richmond, CA). Bands on the dried gel were visualized by autoradiography using Type XAR-5 film (Eastman-Kodak); exposure time was 2-5 hours.

5

(i) Figure 1

Figure 1 depicts an autoradiograph of a non-denaturing polyacrylamide gel which contained MP Oligomer sequence 2100, its corresponding perfectly complementary RNA Oligomer and approximately 50,000 dpm of $^{32}$P-labelled RNA for visualization of the bands by autoradiography. In this experiment, the MP-oligomer had a single phosphate diester linkage at the 5′-end and thus was slightly different from the compound described in Example 1. The gel was run at 6°C.

15 Lane 1: MP oligomer = 3 X 10$^{-9}$ M, RNA = 1 X 10$^{-9}$ M; 
Lane 2: MP oligomer = 3 X 10$^{-9}$ M, RNA = 1 X 10$^{-9}$ M; 
Lane 3: MP oligomer = 3 X 10$^{-7}$ M, RNA = 1 X 10$^{-7}$ M; 
Lane 4: MP oligomer = 2 X 10$^{-9}$ M, RNA = 1 X 10$^{-9}$ M; 
Lane 5: MP oligomer = 2 X 10$^{-8}$ M, RNA = 1 X 10$^{-8}$ M; 
Lane 6: MP oligomer = 2 X 10$^{-7}$ M, RNA = 1 X 10$^{-7}$ M; 
Lane 7: MP oligomer = 1 X 10$^{-9}$ M, RNA = 1 X 10$^{-9}$ M; 
Lane 8: MP oligomer = 1 X 10$^{-8}$ M, RNA = 1 X 10$^{-8}$ M; 
Lane 9: MP oligomer = 1 X 10$^{-7}$ M, RNA = 1 X 10$^{-7}$ M.

a = Bands corresponding to triple-stranded complexes;  
b = bands corresponding to double-stranded complexes;  
c = bands corresponding to single-stranded RNA.

Three distinct bands were visualized in each lane on the autoradiograph, corresponding to single-stranded RNA (lower band), 1:1 MP:RNA double-strand (middle band), and 2:1 MP:RNA triple strand (upper band). Lanes which contained MP and RNA at 1:1 molar ratios had upper and lower bands of roughly equal intensities with very faint middle bands. This indicated that the upper band indeed corresponds to a 2:1 MP:RNA triple strand and that the 1:1 MP:RNA double strand (middle band) is significantly less stable than the triple strand. Lanes
which contained MP and RNA at 2:1 and 3:1 molar ratios had predominant upper bands and very faint middle bands; lower bands were almost completely absent, particularly for the samples which contained RNA in excess of $10^{-8}$ M. Since three distinct bands were observed and the lower bands disappeared at 2:1 and 3:1 stoichiometries, it was concluded that the upper bands corresponded to 2:1 MP:RNA triple-stranded complexes. Furthermore, the relative intensities indicated that the triple-strand complexes are significantly more stable than double-stranded complexes.

(ii) Figure 2

Gel-shift analyses with MP oligomer G2100 and its complementary RNA oligomer R39 were also run at 37°C. Figure 2 depicts an autoradiograph of the resulting non-denaturing polyacrylamide gel. Conditions were the same as described for Figure 1 except that the gel was run at 37°C. a = Bands corresponding to triple-stranded complexes; b = bands corresponding to single-stranded RNA. Note that there are no bands corresponding to double-stranded complexes visible on this autoradiograph.

At this temperature (37°C), the middle bands corresponding to 1:1 MP:RNA double stranded complexes were absent, but the relative intensities of upper bands corresponding to 2:1 MP:RNA triple strands were essentially unchanged compared to the data obtained at 6°C. This indicated that the triple-stranded complex formed between MP oligomer G2100 and its complementary RNA oligomer R39 is much more stable than the double-stranded complex and that it has a very slow off-rate at 37°C.

Similar gel-shift analyses of the type just described were also performed with Set #2 and Set #3, corresponding to MP oligomers and RNA oligomers G2101 & R289 and G2106 & R84, respectively. Gels run at 6°C
again demonstrated that 2:1 MP:RNA triple stranded complexes are formed with each oligonucleotide set. Set #2 also exhibited very prominent upper bands (2:1 MP:RNA triple strands) in gels run at 37°C.

(iii) Figure 3

Figure 3 depicts an autoradiograph of a non-denaturing polyacrylamide gel. The samples contained either of MP oligomer sequences 2100, 2101 and 2106 together with their corresponding perfectly complementary RNA oligomers and approximately 50,000 dpm of $^{32}$P-labelled RNA for visualization of the bands by autoradiography. In this experiment, each of the MP oligomers contained a single phosphate diester linkage at their 5'-ends and therefore are slightly different in composition from the oligomers described in Example 1.

Lane 1: RNA complement to sequence 2100 alone at 1 X $10^{-4}$ M; Lane 2: MP sequence 2100 at 3 X $10^{-3}$ M plus its RNA complement at 1 X $10^{-4}$ M; Lane 3: MP sequence 2100 at 1 X $10^{-4}$ M plus its RNA complement at 1 X $10^{-4}$ M; Lane 4: RNA complement to sequence 2101 alone at 1 X $10^{-4}$ M; Lane 5: MP sequence 2101 at 3 X $10^{-3}$; Lane 6: MP sequence 2101 at 1 X $10^{-3}$ M plus its RNA complement at 1 X $10^{-4}$ M; Lane 7: RNA complement to sequence 2106 alone at 1 X $10^{-4}$ M; Lane 8: MP sequence 2106 at 3 X $10^{-3}$ M plus its RNA complement at 1 X $10^{-3}$ M; Lane 9: MP sequence 2106 at 1 X $10^{-3}$ M plus its RNA complement at 1 X $10^{-4}$ M. a = Bands corresponding to triple-stranded complexes; b = bands corresponding to single-stranded RNA.

(c) Thermal denaturation profiles: The stabilities of triple stranded complexes formed between two MP oligomers and a complementary RNA oligomer were determined by thermal denaturation analysis. Solutions were prepared for analysis as follows: 2.4 µM MP oligomer, 1.2 µM RNA oligomer (2:1 mole ratio MP:RNA) in 10 mM potassium phosphate, 0.1 M sodium chloride, 0.03% potassium sarkosylate, 0.1 mM EDTA, pH 7.2, final volume
= 1 mL. Each solution was heated to 80°C and allowed to cool to 4°C over a period of about 4 hours. The solutions were then transferred to quartz cuvettes (1 cm pathlength) and placed in a Varian Model Cary 3E spectrophotometer equipped with a temperature control module interfaced to an IBM compatible PC computer. Temperature was varied from 5°C to 80°C at a rate of 0.5 °C/min. and absorbance was measured continuously at 260 nm. Plots of A₂₆₀ versus temperature revealed single monophasic transitions for each of the oligomer sets described in this example. The melting temperatures (Tₘ) at which half of each complex had dissociated to single strands were 52°C and 56°C for Sets #1 and #2, respectively. Thus, the complex formed with randomized all-purine MP oligomer G2101 was more stable than that formed with alternating all-purine MP oligomer G2100.

Thermal denaturation profiles for MP oligomer sequences 2100 (-- -- -- -- -- -- -- --), 2101 (-- -- -- -- -- -- -- --), 2102 (-- -- -- -- -- -- -- --) and 2106 (-- -- -- -- -- -- -- -- --) in the presence of their corresponding, perfectly complementary RNA target oligomers. Solutions of 2.4 μM MP oligomer and 1.2 μM complementary RNA oligomer were prepared in 20 mM potassium phosphate, 0.1 M sodium chloride, 0.03% potassium sarkosylate, 0.1 mM EDTA, pH 7.2, final volume = 1 mL. Each solution was heated to 80°C and then cooled slowly to 4°C over a period of about 4 hours. The resulting solutions were then monitored by absorbance at 260 nm as a function of temperature (ramp = 1.5°C/minutes) using a Varian Model Cary 3E UV/Vis spectrophotometer containing a temperature-controlled 6 X 6 cell module and interfaced to an IBM compatible PC computer. The increase in absorbance with temperature is proportional to the number of oligomer strands which have denatured. The Tₘ is defined as the point where 50% of the strands have denatured.
(d) **Conclusions**

The data presented in Example 1 indicate that all-purine MP oligomers containing a 50:50 mixture of adenines and guanines are capable of forming 2:1 MP:RNA triple stranded complexes with their complementary RNA oligomers.

We can deduce the polarity of the two MP oligomer strands in these triple-stranded complexes as follows. A parallel orientation permits each of the bases in the third strand to participate in hydrogen bonding. On the other hand, not all of the bases can hydrogen bond in an antiparallel orientation. For example, an antiparallel orientation for two strands of MP oligomer G2100 would require a single-base overhang at the end of the third strand. An antiparallel orientation for two strands of either MP oligomer G2101 or MP oligomer G2106 would require a three-base overhang at the end of the third strand. Therefore, we would predict that an antiparallel configuration would be significantly less stable, especially for triple stranded complexes formed with the randomized MP oligomers G2101 and G2106. In fact, gel-shift analysis showed that each of these MP oligomers forms a triple stranded complex with its complementary RNA oligomer which is much more stable than the corresponding double stranded complex. Furthermore, thermal denaturation profiles showed that MP oligomer G2106 forms a more stable triple stranded complex than MP oligomer G2101. These observations indicate that the two MP oligomers in each triple stranded complex just described are binding in a parallel orientation.
Example 2

Triple Helix Complex Formation With Methylphosphonate Oligomers and Complementary RNA Oligomers Containing Alternating Purines and Pyrimidines

The following MP oligomers and complementary RNA oligomers were examined by UV mixing curve analysis according to the procedure described in Example 1.

Set #4:  
G2019  5'-GTGCTGTGTGTGTGT-3' (MP oligomer)  
R138   3'-CACACACACACACACA-5' (RNA oligomer)

Set #5:  
G2018  5'-ACACACACACACACAC-3' (MP oligomer)  
R139   3'-UGUGUGUGUGUGUGUGUG-5' (RNA oligomer)

The molar extinction coefficients, $\epsilon_{254}$ (M$^{-1}$cm$^{-1}$) determined for G2019 and G2018 were determined to be 12.0 X 10$^4$ and 15.01 X 10$^4$, respectively. The molar extinction coefficients determined for R138 and R139 were determined to be 12.3 X 10$^4$ and 11.4 X 10$^4$, respectively.

UV mixing curve analysis for Set #4 showed a single transition point at 15°C, $X_{mp} = 0.67$, indicating a single 2:1 MP:RNA triple-stranded complex. This demonstrates the possibility of forming a triple stranded complex with two MP oligomers and a complementary RNA oligomer where each strand contains alternating purines and pyrimidines.

UV mixing curve analysis for Set #5 also showed a single transition point at 15°C, but in this case it occurred at $X_{mp} = 0.33$, indicating a single 2:1 RNA:MP triple-stranded complex. This demonstrates the possibility of forming a triple stranded complex with two RNA oligomers and a complementary MP oligomer where each strand contains alternating purines and pyrimidines.

The conclusion from Example 2 is that MP oligomers which contain alternating purines and pyrimidines are capable of forming triple stranded complexes with complementary RNA oligomers.
Example 3
Triple Helix Complex Formation Between an MP Oligomer Containing a Random Mixture of Purines and Thymidine and its Complementary RNA Oligomer

The following set of MP oligomer and complementary RNA oligomer was examined by UV mixing curve analysis according to the procedure described in Example 1.

Set #6: G2102 5'-AGATAGGGAGTGGGAA-3' (MP oligomer)
        R291 3'-UCUAUCCUCACCCUU-5' (RNA oligomer)

The molar extinction coefficients, ε_{225} (M^{-1}cm^{-1})
determined for G2102 and R291 were determined to be
16.82 x 10^4 and 11.37 x 10^4, respectively.

UV mixing curve analysis for Set #6 showed a single transition point at 15°C, X_{MP} = 0.67, indicating a single
2:1 MP:RNA triple-stranded complex. This demonstrates the possibility of forming a triple stranded complex with two MP oligomers and a complementary RNA oligomer where the MP oligomer contains a mixture of purines and thymidine.

The data presented in Example 3 is further evidence for a parallel configuration between the two strands of MP oligomer in the 2:1 MP:RNA triple strand. Significantly less bases in the third strand would be capable of hydrogen bonding in an antiparallel configuration.

Example 4
Triple Helix Complex Formation Between Several Different MP-Oligomers Having Predominantly Purine-Rich Sequences and Their Corresponding RNA Targets

This example describes a gel-shift experiment which was done to demonstrate a triple helix complex between several different Oligomers having a predominantly purine sequence and their corresponding RNA targets.

The following sets of methylphosphonate Oligomers and their complementary RNA targets were compared in a
gel-shift experiment similar to the one described in Example 1:

G2100 5'-'AGA-GAG-AGA-GAG-AGA-G-3' (methylphosphonate)  
R39   3'-UCU-CUC-UCU-CUC-UCU-C'-5' (ribooligonucleotide)  

5'-'AGA-AAG-GGA-GAG-GGA-A-3' (methylphosphonate)  
R289  3'-UCU-UUC-CCU-CUC-CCU-U-5' (ribooligonucleotide)  

G2102 5'-'AGA-TAG-GGA-GTG-GGA-A-3' (methylphosphonate)  
R291  3'-UCU-AUC-CCU-CAC-CCU-U-5' (ribooligonucleotide)  

G2104 5'-'AGA-AAG-GGA-CAG-GGA-A-3' (methylphosphonate)  
10 R293  3'-UGU-UUC-CCU-GUC-CUC-U-5' (ribooligonucleotide)  

G2106 5'-'GAG-GGA-AAG-AGA-AAG-G-3' (methylphosphonate)  
R84   3'-CUC-CCU-UUC-UUC-UUC-C'-5' (ribooligonucleotide)  

Molar extinction coefficients, \( \epsilon_{260} \) (M\(^{-1}\).cm\(^{-1}\)) for Oligomers G2104 and R293 were determined to be 16.51 X 10\(^4\) and 13.78 X 10\(^4\), respectively, according to the protocol given in Example 1.

Solutions containing 1 X 10\(^{-5}\) M MP-oligomer and 5 X 10\(^{-6}\) M RNA-Oligomer (2:1 mole ratios) were prepared in 1.5 mL polypropylene microcentrifuge tubes along with 10 mM potassium phosphate, 0.03% potassium sarkosyltate, 0.1 mM EDTA, pH 7.2 and 250,000 cpm of \(^{32}\)P-labelled RNA-oligomer (approximately 0.5 pmole), total volume = 50 \( \mu \)L. The tubes were heated to 80\(^\circ\)C, cooled to 4\(^\circ\)C over a period of about four hours, and then kept at 4\(^\circ\)C for about 48 hours. Next, 5 \( \mu \)L aliquots were added to separate tubes containing 5 \( \mu \)L of 50% glycerol, 1 X TBE buffer, 0.1% bromphenol blue on ice. These samples were then analyzed by non-denaturing gel electrophoresis at 6 according to the protocol described in Example 1. The original samples were then equilibrated at 30\(^\circ\)C for four hours. Next, 5\( \mu \)L aliquots were added to separate tubes containing 5 \( \mu \)L of 50% glycerol, 1 X TBE buffer, 0.1% bromphenol blue which had been equilibrated at 30\(^\circ\)C in a water bath. These samples were analyzed by non-denaturing gel electrophoresis at 30\(^\circ\)C.

Examination of the autoradiograph from the gel which had been run at 6\(^\circ\)C revealed that each of the sets
of oligomers analyzed in this example formed predominantly triple helix complexes with their RNA targets. There was a faint band corresponding to double-stranded complex in the lane corresponding to oligomer 2104, however. The autoradiograph corresponding to the gel run at 30°C revealed predominantly triple helix complexes for Oligomers 2100, 2101 and 2102. Any complexes formed with Oligomer 2104 and its RNA target had mostly dissociated either prior to or during gel electrophoresis at 30°C. The triple helix complex formed with oligomer 2106 and its RNA target had mostly dissociated, but was still evident as a distinct upper band on the gel. In no case were bands corresponding to double-stranded complexes evident in the gel run at 30°C. Based on the intensities of bands corresponding to triple helix complexes and single-stranded RNA resulting from both gels, a relative order of the stabilities of each triple helix complex was determined as follows:

G2101:R289>G2100:R39>G2102:R291>G2106:R84>G2104:R293

The three all-purine MP-oligomers described in this example each formed triple helix complexes with their target RNA-Oligomers, but with different relative stabilities. The sequences of MP-Oligomers G2102 and G2104 are nearly identical to the sequence of G2101 except that they contain either two thymidines replacing two adenines or two cytidines replacing two guanines, respectively. As illustrated in this example, each of MP-Oligomers G2101, G2102 and G2104 is capable of forming a triple helix complex with their target RNA. However, the data suggests that replacing two adenines with two thymidines is somewhat destabilizing, and that replacing two guanines with two cytidines is more destabilizing with respect to triple helix formation.
Due to the random distribution of purines and asymmetric replacement of two pyrimidines in each of sequences G2102 and G2104, an antiparallel triple helix complex is unlikely, since there would be significantly less possible hydrogen-bonding interactions in the antiparallel motif than in the parallel motif. Thus, this example presents further evidence for a parallel binding motif in triple helix complexes formed with predominantly all-purine MP-Oligomers containing up to about 12% pyrimidines (2 out of 16).

Example 5
Inhibition of Translation With Triple Helix Complex Forming Modified Oligonucleotides

Sequence specific inhibition of an mRNA by formation of a triple helix complex was demonstrated by the following procedure.

The target site for the alternating AG methylphosphonate 16 mer was cloned immediately 5' of the translation initiation site in the chloramphenicol acetyltransferase (CAT) gene (Gorman et al., Mol. and Cell. Bio. (1982) 2:1044-1051) in a T7 transcription vector by standard cloning techniques (Molecular Cloning Sambrook et al. (1989) CSH Laboratory Press). Capped mRNA was transcribed with T7 polymerase (Melton, D.A. et al. (1984) Nuc. Acids Res. 12:7035-7056) and beta-globin RNA served as an internal control, to demonstrate the specificity of this translation inhibition. Reticulocyte lysates, unlabelled amino acids, and translation buffers were obtained from Life Technologies. A mixture of ~80 ng of CAT mRNA per reaction containing the AG alternating target site (Alternating stretch of CU) and ~30 ng of globin RNA per reaction along with buffers, amino acids, 35-S-Methionine (DuPont NEN, Boston, MA), and rabbit reticulocyte lysate were combined on ice to form the
standard translation mix (Polayes, D.A., (1991) Focus 12:4). This mix was aliquoted into tubes containing the methylphosphonate oligomers dissolved in water or 20 mM potassium acetate to give final concentrations after addition of the mix of 25 μM, 3 μM or 0.3 μM. The translation reactions were allowed to proceed for 60 minutes, then 1.5 μg of RNase A was added and the reaction continued for 15 minutes. Gel loading buffer was added, and the samples were electrophoresed in 10% Acrylamide/tricine buffered pre-cast protein gels (Novex, San Diego, CA). The gels were fixed in 10% acetic acid 40% methanol, dried, and exposed to X-ray film for 12–72 hours.

The resulting autoradiograph is shown in Figure 5. The upper band is the translation product protein of the targeted CAT mRNA containing the alternating CU 18 nt site adjacent to the initiator codon. The lower band is the protein product of the internal control globin mRNA. Addition of the triplex forming alternating AG methylphosphonate oligomer resulted in a dramatic reduction in the translation of the CAT gene, but not the globin gene. This example demonstrates that the triplex complex was able to specifically block the translation of the target gene. This inhibition is greater that the inhibition seen with unmodified DNA of similar length (Maher, LJ and Dolnick, BJ Nucleic Acids Res. (1988) 16:3341–3355).

Example 6
Inhibition of Reverse Transcription with Triple Helix Complex Forming Modified Oligonucleotides

Sequence specific inhibition of reverse transcription was demonstrated by the following procedure.

The target site for the triple helix forming methylphosphonates 16 mer (MF oligo 2100, 2101 or 2102,
see Example 4 for sequences) was cloned immediately 5' of the translation initiation site in the chloramphenicol acetyltransferase (CAT) gene (Gorman, et al., Mol., and Cell Bio. (1982) 2:1044-1051) in a T7 vector by standard cloning techniques (Molecular Cloning Sambrook, et al. (1989) CS Laboratory Press). Capped mRNA was transcribed with T7 polymerase (Melton, D.A. et al. (1984) Nuc. Acids Res. 12:7035-7056). Approximately 1 µg of RNA with 0.5 µg of DNA primer (5' CCATTGGGATATATC) was added to the 10 micromolar MP oligonucleotide in 20 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 0.03% sarkosyl, 100 mM NaCl. The mixture was heated to 70 degrees C., cooled to 4 degrees C., and incubated overnight. AMV reverse transcriptase (Promega biotech) was added with 50 mM Tris 3 mM MgCl, and 1 mM of dNTPs, and ^35S dATP (DuPont NEN, Boston, MA). The resulting mixture was incubated for 1 hour at 30-37 degrees C., and then applied to 8.0% acrylamide/Urea gel (Molecular Cloning, Sambrook et al. (1989) CSH Laboratory Press). The gels were fixed in 10% acetic acid/10% methanol and then dried. The gel was exposed to X-ray film for 3 days to give an autoradiograph.

Ordinarily reverse transcriptase will begin making a RNA template driven DNA copy beginning at the priming site of the added DNA Oligomer (MP Oligomer does not act as a primer) and ending as the polymerase reaches the end of RNA template (a full length product). Blocking of the progression of the polymerase by a MP Oligomer will result in a truncated transcript corresponding in length to the distance between the priming Oligomer and the blocking Oligomer.

The predicted ~105 nt full length product was observed in the reverse transcription control lanes in which no MP Oligomer was added. When MP oligos 2100, 2101 or 2102 were added to reactions containing their respective target RNAs the predicted ~50 nt truncated
fragments were observed. The triple helix forming Oligomers 2100 and 2101 gave almost complete conversion of ~105 nt fragment to the ~50 nt fragment which demonstrated specific blocking of the reverse transcriptase enzyme. The 2102 MP Oligomer gave partial conversion of the ~105 nt fragment to the ~50 nt fragment, which demonstrated partial blocking of the reverse transcriptase enzyme.

Example 7
Preparation of Oligoribonucleosides
The oligoribonucleotides described in Examples 1 to 4 were synthesized using the following procedures:

The oligoribonucleotides were synthesized using 5'-O-dimethoxytrityl-2'-O-tert-butyldimethylsilyl-3'-O-N,N-diisopropyl-β-cyanoethylphosphoramidite nucleosides (purchased from either Millipore or Peninsula Laboratories). The syntheses were done on a 1 μmole scale with a Milligen 8750 automated DNA synthesizer using standard Milligen phosphoramidite procedures with the exception that the coupling times were extended to 12 minutes to allow adequate time for the more sterically hindered 2'-O-tert-butyldimethylsilyl RNA monomers to react. The syntheses were begun on control-pore glass bound 2'-O-tert-butyldimethylsilyl ribonucleosides purchased from Peninsula Laboratories. All other oligonucleotide synthesis reagents were as described in Milligen's standard protocols.

After synthesis, the oligonucleotides were handled under sterile, RNase-free conditions. Water was sterilized by overnight treatment with 0.5% diethylpyrocarbonate followed by autoclaving. All glassware was baked for at least 4 hours at 300°C.

The oligonucleotides were deprotected and cleaved from support by first treating the support bound oligomer with 3/1 ammonium hydroxide/ethanol for 15
hours at 55°C. The supernatant, which contained the oligonucleotide, was then decanted and evaporated to dryness. The resultant residue was then treated with 0.6 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran (which contained 5% or less water) for 24 hours at room temperature. The reaction was quenched by the addition of 0.6 mL of aqueous 2 M triethylammonium acetate, pH 7. Desalting of the reaction mixture was accomplished by passing the solution through a Bio-Rad 10DG column using sterile water. The desalted oligonucleotide was then dried.

Purification of the oligoribonucleotides was done by polyacrylamide gel electrophoresis (PAGE) containing 15% 19/1 polyacrylamide/bis-acrylamide and 7 M urea using standard procedures (See, Maniatis, T. et al., Molecular Cloning A Laboratory Manual, pages 184-185 (Cold Spring Harbor 1982)). The gels were 20 cm wide by 40 cm long and 6 mm in width. The oligoribonucleotides (60 OD Units) were dissolved in 200 μL of water containing 1.25% bromophenol blue and loaded onto the gel. The gels were run overnight at 300 V. The product bands were visualized by UV backshadowing, excised, and the product eluted with 0.5 M sodium acetate overnight. The product was desalted using a Waters C18 Sep-Pak cartridge with the manufacturer supplied protocol. The product was then kinased and analyzed by PAGE.

Example 8
Inhibition of Translation With Triple Helix Complex Forming Modified Oligonucleotides

Sequence specific inhibition of an mRNA by formation of a triple helix complex was demonstrated by the following procedure.

The target site for the alternating AG methylphosphonate 16 mer was cloned immediately 3' of the translation initiation site in the chloramphenicol
acetyltransferase (CAT) gene (Gorman et al., Mol. and Cell. Bio. (1982) 2:1044-1051) and inserted into the Hind III/Not I sites in a T7 transcription vector (pRC-CMV (InVitrogen, San Diego, CA)) by standard cloning techniques (Molecular Cloning Sambrook et al. (1989) CSH Laboratory Press). A CAT containing template (pBR325, Life Technologies, Gaithersberg, MD) was used to obtain the CAT coding region. Capped mRNA was transcribed with T7 polymerase (Melton, D.A. et al. (1984) Nuc. Acids Res. 12:7035-7056). A control CAT sequence that did not contain the (AG)$_8$ target site and encoded a truncated CAT protein and beta-globin RNA served as internal controls, to demonstrate the specificity of this translation inhibition. MP(AG)$_8$ or DE(AG)$_8$ oligomers were included in the translation mix as indicated in Figure 7. Reticulocyte lysates, unlabelled amino acids, and translation buffers were obtained from Life Technologies. A mixture of ~30 ng of CAT mRNA per translation reaction and ~30 ng of globin RNA per reaction along with buffers, amino acids, $^{35}$S-Methionine (DuPont NEN, Boston, MA), and rabbit reticulocyte lysate were combined on ice to form the standard translation mix (Poyales, D.A., (1991) Focus 13:4). This mix was aliquoted into tubes containing the methylphosphonate oligomers dissolved in water or water alone to give final concentrations of translation constituents including 68 mM potassium acetate (20 mM added, 48 mM in mix). Oligomer concentration after addition of the mix was 1 $\mu$M, 0.1 $\mu$M or 0.01 $\mu$M as indicated in Figure 7. The translation reactions were allowed to proceed for 60 minutes, then 1.5 $\mu$g of RNase A was added and the reaction continued for 15 minutes. Gel loading buffer was added, and the samples were electrophoresed in 10% polyacrylamide/tricine buffered pre-cast protein gels (Novex, San Diego, CA). The gels were fixed in 10%
acetic acid 40% methanol, dried, and exposed to X-ray film for 12-72 hours.

The resulting autoradiograph is shown in Figure 7. The upper band is the translation product protein of the targeted CAT mRNA containing the alternating CU 16 nt site adjacent to the initiator codon. The middle band is the protein product of the internal control globin mRNA. The lower band is the protein product of the CAT control. Addition of the triplex forming alternating (AG)₈ methylphosphonate oligomer resulted in a dramatic reduction in the translation of the CAT gene, but not the globin gene or the CAT control.

This example demonstrates that the triplex complex was able to specifically block the translation of the target gene. MP-(AG)₈ specifically inhibits protein translation from the CAT(CU)₈ mRNA target, whereas DE-(AG)₈ shows no inhibition at any of the concentrations tested in the absence of RNase H. RNase H was added in one lane as a positive control to confirm that the DE(AG)₈ oligonucleotide has indeed hybridized to the CAT-(CU)₈ mRNA. This result is consistent with the binding of DE-(AG)₈ as a heteroduplex rather than a triple-stranded complex, since RNase H would not be expected to act on a triple stranded substrate.

Example 9
Inhibition of Translation With Triple Helix Complex Forming Modified Oligomers Having a Scrambled (AG) Sequence

A target site for the scrambled AG methylphosphonate 16-mer "(AG)-S1"; Sequence: 5'- AGAAAGGGAGAGGAA-3') was cloned and inserted into a T7 transcription vector as described in Example 8. Capped mRNA was transcribed as described in Example 8. The beta-globin and CAT(CU)₈ RNAs described in Example 8 were also used in the translation mixes. A MP oligomer
having the same sequence as MP-(AG)-S1 but with two thymidine based replacing two adenine bases was prepared and tested as a two-base mismatch ("2mm") control. MP-(AG)-S1, 2mm or MP(AG)\textsubscript{8} oligomers were included in the translation mixes as indicated in Figure 8.

Translation reactions were performed as described in Example 8. The protein products were separated by electrophoresis and exposed to film as described in Example 8.

The resulting autoradiograph is shown in Figure 8. The upper band is the translation product of the CAT-(CU)-S1 target mRNA. The lower bands are protein products of \(\beta\)-globin mRNA (included as an internal control) and the CAT(CU)\textsubscript{8} mRNA, respectively.

As may be seen from Figure 8, the scrambled MP-(AG)-S1 oligomer inhibited translation of its corresponding CAT(CU)-S1 target mRNA. At 10 \(\mu\)M, MP-(AG)-S1 also caused some non-specific inhibition, as evidenced by the slightly fainter control band. The 2mm oligomer did not inhibit CAT(CU)-S1 protein synthesis at any of the concentrations tested, further demonstrating sequence specific inhibition by the fully complementary MP oligomers. Additionally, MP-(AG)-S1 and MP-(AG)\textsubscript{8} only inhibited their intended complementary mRNA targets (CAT-(CU)-S1 and CAT-(CU)\textsubscript{8}, respectively).

Example 10

Triple-Strand Formation With All-Purine MP Oligomers Directed to a Double-Stranded DNA Target

The following example demonstrates that all-purine methylphosphonate oligomers are capable of forming triple-stranded complexes with double-stranded DNA targets.

The DNA target used in this example (sequence #2562) has complementary 3′- and 5′-ends. This causes the target to form a hairpin structure:
Oligomer No.

2562-1
5'-AAA-AAA-GAG-GAG-GGA T
3'-TTT-TTT-CTC-CTC-CCT T

Thermal denaturation analysis of this oligomer confirmed the hairpin structure. Thus, monitoring $A_{260}$ versus temperature for 2562-1 as described in Example 1 gave a single melt transition with a Tm of 74°C.

The following methylphosphonate (MP) oligomers were studied for their ability to hybridize to this DNA hairpin sequence:

<table>
<thead>
<tr>
<th>Sequence Designation</th>
<th>MP Oligomer Sequence No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiparallel AG</td>
<td>2317-1</td>
<td>5'-AGG-GAG-GAG-AAA-AAA-3'</td>
</tr>
<tr>
<td>Parallel AG</td>
<td>2318-1</td>
<td>5'-AAA-AAA-GAG-GAG-GGA-3'</td>
</tr>
</tbody>
</table>

Each of these oligomers was mixed in a 1:1 molar ratio target Oligomer No. 2562-1, annealed and analyzed by thermal denaturation analysis as described in Example 1. Biphasic melt transitions were observed for each oligomer hybridized to the DNA target. Based on control experiments with the MP oligomers and DNA target run separately, the first transition was shown to correspond to denaturation of the triple stranded MP/DNA complex, whereas the second transition was shown to correspond to denaturation of the DNA hairpin. The Tms taken from the first transitions are summarized below:

<table>
<thead>
<tr>
<th>Sequence Designation</th>
<th>MP Oligomer Sequence No.</th>
<th>Tm (1:1 with DNA Target 2562-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiparallel AG</td>
<td>2317-1</td>
<td>35.9°C</td>
</tr>
<tr>
<td>Parallel AG</td>
<td>2318-1</td>
<td>49.1°C</td>
</tr>
</tbody>
</table>
This data shows that both the antiparallel and parallel MP oligomers can form triple-stranded complexes with the double-stranded DNA hairpin. The Tm values indicate that the parallel AG oligomer 2318-1 binds more tightly to the DNA hairpin than the antiparallel AG oligomer 2317-1.

Example 11
Triple Helix Complex Formation With Two Methylphosphonate Oligomers and One Complementary Oligoribonucleoside

Triple helix formation with 2:1 MP:RNA oligomers was demonstrated using thermal denaturation methods such as described in Example 1 using a Varian Model Cary 3E Spectrophotometer. Temperature was varied from 5 to 80 or 90°C at a rate of 0.5 to 1°C/min. The sequences of exemplary triple helix forming MP-oligomers and their TM’s are set forth in the Table below:

<table>
<thead>
<tr>
<th>Seq. No.</th>
<th>Sequence</th>
<th>Tm, 2:1 MP:RNA(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>GGG-GG-GAG-GGA-GG</td>
<td>69.6</td>
</tr>
<tr>
<td>2206</td>
<td>GAG-GAG-GAG-GAG-GAA-GG</td>
<td>57.6</td>
</tr>
<tr>
<td>2278</td>
<td>AGA-AGG-AGG-AGA-GAA</td>
<td>55.8</td>
</tr>
<tr>
<td>2101</td>
<td>AGA-AAG-GGA-GAG-GGA-A</td>
<td>55.6</td>
</tr>
<tr>
<td>2120</td>
<td>AGA-AAG-GGA-GAG-GGA-A</td>
<td>55</td>
</tr>
<tr>
<td>2100</td>
<td>AGA-GAG-AAG-AAG-AAG-G</td>
<td>52.9</td>
</tr>
<tr>
<td>2103</td>
<td>AGA-AGG-GGA-GGA-GGA-A</td>
<td>52.3</td>
</tr>
<tr>
<td>2102</td>
<td>AGA-AGG-GGA-GGA-A</td>
<td>47.6</td>
</tr>
<tr>
<td>2149</td>
<td>GAG-GGA-GAG-GGA-GAG</td>
<td>42</td>
</tr>
<tr>
<td>2106</td>
<td>GAG-GGA-AAG-AAG-AAG-G</td>
<td>40.3</td>
</tr>
<tr>
<td>2104</td>
<td>ACA-AAG-GGA-CAG-GGA-A</td>
<td>36.6</td>
</tr>
</tbody>
</table>
Claims

1. A method of detecting or recognizing a nucleic acid having a single stranded target sequence by formation of a triple helix complex by binding together a First Strand, a Second Strand and a Third Strand, wherein the target sequence is one of the strands and the other strands are Oligomers which are optionally covalently linked, which method comprises binding the First Strand, with Second and Third Stands which are substantially identical in nucleoside sequence to each other and one of which is sufficiently complementary to the First Strand to bind thereto by Watson-Crick base pairing.

2. A method according to claim 1 wherein the First Strand is the target sequence.

3. A method according to claim 2 wherein, in the triple helix complex, the Second Strand and the Third Strand bind parallel to each other and anti-parallel to the First Strand.

4. A method according to claim 3 wherein the Second Strand binds to the First Strand by Watson-Crick base pairing and wherein the Third Strand hydrogen bonds with and binds to both the First Strand and the Second Strand to give the triple helix complex.

5. A method according to claim 1 wherein the Second Strand is the target sequence.

6. A method according to claim 1 wherein the Third Strand is the target sequence.

7. A method of inhibiting or altering expression of a nucleic acid having a single-stranded target
sequence by binding together a First Strand, a Second Strand and Third Strand to give a triple helix complex, wherein one of the strands is the target sequence and the other strands are Oligomers which are optionally covalently linked which method comprises:

contacting the First Strand with Second and Third Strands which have nucleoside sequences substantially identical to each other and one of which is sufficiently complementary to the First Strand to be able to bind thereto by Watson-Crick base pairing.

8. A method according to claim 7 wherein the First Strand is the target sequence.

9. A method according to claim 8 wherein in the triple helix complex the Second Strand and the Third Strand bind parallel to each other and anti-parallel to the target sequence.

10. A method according to claim 9 wherein the Second Strand binds to the target sequence by Watson-Crick base pairing and wherein the Third Strand hydrogen bonds with both the target sequence and the Second Strand to give the triple helix complex.

11. A method according to claim 7 wherein the Second Strand is the target sequence.

12. A method according to claim 7 wherein the Third Strand is the target sequence.

13. A method according to any of claims 1 to 10 wherein the target sequence comprises a predominately pyrimidine nucleoside sequence.
14. A method according to claim 13 wherein the Oligomers are substantially neutral Oligomers.

15. A method according to claim 14 wherein the substantially neutral Oligomers are methylphosphonate Oligomers.

16. A method according to claim 14 wherein the target sequence comprises about 85 percent or greater pyrimidine nucleosides.

17. A method of forming a stable triple helix complex by binding together a First Strand, a Second Strand and a Third Strand wherein one of the strands is a target sequence of single stranded RNA and the two other strands are Oligomers and wherein the Second and Third Strands have the same strand polarity, have substantially identical nucleoside sequences, and are independently substantially complementary to the target sequence.

18. A method according to claim 17 wherein the First Strand comprises predominantly pyrimidine nucleosides and the Second and Third Strands comprise predominantly purine nucleosides.

19. A method according to claim 17 wherein the First Strand is the target sequence.

20. A method according to claim 19 wherein the target sequence comprises a predominantly pyrimidine target sequence and the Oligomers comprise predominantly purine nucleosides.

21. A method according to claim 20 wherein the Oligomers are substantially neutral Oligomers.
22. A method according to claim 21 wherein the substantially neutral Oligomers are methylphosphonate Oligomers.

23. A method according to any of claims 15 to 18 wherein in the triple helix complex the Oligomers bind parallel to each other and anti-parallel to the target sequence.

24. A method according to claim 23 wherein the bases of the nucleosides of the Oligomers hydrogen bond with each other and with the bases of the nucleosides of the target sequence.

25. A method according to claim 24 wherein the Oligomers are substantially neutral Oligomers.

26. A method according to claim 25 wherein the substantially neutral Oligomers are methylphosphonate Oligomers.

27. A method of detecting or recognizing a nucleic acid having a single stranded target sequence which comprises binding a First Strand, a Second Strand and a Third Strand wherein one of the strands is the target sequence and the other strands are Oligomers which are optionally covalently linked whereby the Second Strand is sufficiently complementary to the First Strand to bind to the First Strand by Watson-Crick base pairing and the Third Strand hydrogen bonds with and binds to both the First Strand and the Second Strand by recognizing both bases of each Watson-Crick base pair, thereby forming a triple helix complex.
28. A method according to claim 27 wherein expression of the single stranded nucleic acid is inhibited or decreased.

29. A method according to claim 27 wherein the Second and Third Strands are parallel to each other and have substantially identical nucleoside sequences.

30. A method according to claim 29 wherein the Oligomers are substantially neutral Oligomers.

31. A method according to claim 30 wherein the substantially neutral Oligomers are methylphosphonate Oligomers.

32. A method of detecting or recognizing a double stranded nucleic acid having a double stranded target sequence which has a sense strand and an antisense strand which comprises contacting the target sequence with a Third Strand having a nucleoside sequence substantially identical to the nucleotide sequence of one strand of the target sequence wherein a base of the Third Strand hydrogen bonds to a corresponding base of each strand of the target sequence to give a triplet and wherein multiple triplets are formed to give a triple helix complex.

33. A method according to claim 32 wherein expression of the single stranded nucleic acid is inhibited or decreased.

34. A method according to claim 33 wherein the Third Strand is parallel to and has a nucleoside sequence substantially identical to either the sense strand or the antisense strand.
35. A method of inhibiting expression of a nucleic acid having a single stranded target sequence by binding together a First Strand, a Second Strand and a Third Strand to give a triple helix complex wherein one of the strands is the target sequence and the other strands are Oligomers which are optionally covalently linked which comprises:

contacting the First Strand with Second and Third Strands which are parallel to each other and have

substantially identical nucleotide sequences to each other wherein the Second Strand is sufficiently complementary to the First Strand to bind to the target sequence by Watson-Crick base pairing and wherein the Third Strand hydrogen bonds with both the Second Strand and the First Strand to give a triple helix complex.

36. A method according to claim 35 wherein the First Strand comprises a predominantly pyrimidine nucleoside sequence.

37. a method according to either claim 35 or 36 wherein the Oligomers are substantially neutral Oligomers.

38. A method according to claim 37 wherein the substantially neutral Oligomers are methylphosphonate Oligomers.
FIG. 1.
SUBSTITUTE SHEET (RULE 26)
FIG. 2.
**FIG. 3.**

SUBSTITUTE SHEET (RULE 26)
FIG. 4

Normalized Absorbance, 260 nm

Temperature (°C)

0 10 20 30 40 50 60 70 80

0.7 0.8 0.9 1

SUBSTITUTE SHEET (RULE 26)
FIG. 7.

(AG)8

0.01μM 0.1μM 1μM
MP DE MP DE MP DE

DE & RNase H

CAT (CU)8 ➔

β-Globin ➔

CAT Control ➔
FIG. 8.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(5) :A61K 48/00; C12Q 1/68; C07H 21/04
   US CL :435/6; 514/44; 536/25.3
   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. : 435/6; 514/44; 536/25.3
   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)
   APS, MEDLINE, BIOSIS
   search terms: triple helix, triplex, gene expression

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X,P</td>
<td>US, A, 5,176,996 (Hogan et al.) 05 January 1993, col. 8, lines 4-17.</td>
<td>32-34</td>
</tr>
<tr>
<td>Y,P</td>
<td></td>
<td>1-31, 35-38</td>
</tr>
</tbody>
</table>

* Special categories of cited documents:
  "A" - document defining the general state of the art which is not considered to be part of particular relevance
  "F" - earlier document published on or after the international filing date
  "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)
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  "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
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  "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

Date of the actual completion of the international search
28 January 1994

Date of mailing of the international search report
FEB 25 1994

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
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Washington, D.C. 20231

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