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Splice-region antisense composition and method

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(71) Applicant(s)
AVI BioPharma, Inc.

(72) Inventor(s)
Iversen, Patrick L. Hudziak, Robert

(74) Agent/Attorney
FB Rice & Co, Level 23 200 Queen Street, Melbourne, VIC, 3000

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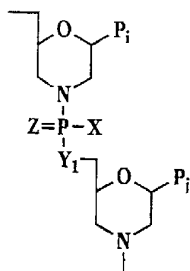
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- (71) Applicant: **AVI BIOPHARMA, INC.** [US/US]; Suite
200, 4575 S.W. Research Way, Corvallis, OR 97333 (US).
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- (72) Inventors: **IVERSEN, Patrick, L.**; 5902 N.W. Fair Oaks
Place, Corvallis, OR 97330 (US). **HUDZIAK, Robert**;
20244 Hwy. 20, P.O. Box 585, Blodgett, OR 97326 (US).
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(54) Title: **SPLICE-REGION ANTISENSE COMPOSITION AND METHOD**

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(57) **Abstract:** Antisense compositions targeted against an mRNA sequence for a selected protein, at a region having its 5' end from 1 to about 25 base pairs downstream of a normal splice acceptor junction in the preprocessed mRNA, are disclosed. The antisense compound is RNase-inactive, and is preferably a phosphorodiamidate-linked morpholino oligonucleotide. Such targeting is effective to inhibit natural mRNA splice processing, produce splice variant mRNAs, and inhibit normal expression of the protein.

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Splice-Region Antisense Composition and Method

Field of the Invention

The present invention relates to therapeutic compositions and methods for inhibiting expression of full-length proteins in cells, and in particular to antisense compositions targeted against an mRNA sequence having its 5' end 1 to about 25 base pairs downstream of a normal splice acceptor junction in a preprocessed mRNA. Such targeting is effective to inhibit natural mRNA splice processing and produce splice variant mRNAs.

Background of the Invention

Inhibition of protein expression by antisense targeting of DNA or RNA coding for the protein has been the subject of extensive study. Many reported procedures have employed phosphorothioate-linked oligonucleotides, which are charged, nuclease-resistant analogs of native DNA. The antisense mechanism involved is based on the activation of RNase, which cleaves the target nucleic acid to which the oligomer is bound. While these compounds have shown high activity, they also tend to show high levels of side effects, i.e. by cleavage of non-target RNA or by non-antisense mechanisms, such as nonspecific binding to proteins.

Another class of antisense oligomers, termed RNase-inactive, do not promote cleavage of bound RNA and are believed to act by sterically blocking the molecular machinery from transcribing, processing, or translating the target sequence. While these compounds tend to produce fewer side reactions, such as nonselective cleavage, than phosphorothioate oligomers, it has generally been necessary to target specific regions of RNA, such as the AUG start codon, for successful inhibition.

More recently, targeting of the splice acceptor junction of nuclear (unspliced) RNA by RNase-inactive oligomers has been reported. Kole and Dominski (U.S. Patent No. 5,665,593) reported suppression of missplicing of β -globin RNA, in order to combat variants of β -thalassemia which result from such aberrant splicing. In this case, the aberrant splice junction was targeted, to direct splicing back to the normal site. RV Giles *et al.*, *Antisense & Nucleic Acid Drug Dev.* 9:213-220 (1999), targeted a splice junction to induce missplicing of *c-myc* mRNA. In each of these cases, the region targeted is still somewhat restricted, in that the antisense oligomer spans the intron/exon splice junction of the pre-mRNA. Due to the advantages accorded by the use of uncharged, RNase-inactive oligonucleotides, a demonstration of further flexibility in targeting would be quite useful.

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Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

1A

Summary of the Invention

In one aspect, the invention provides an antisense compound, and a corresponding method of inhibiting normal splicing of preprocessed RNA in a eukaryotic cell, by contacting the cell with such an antisense compound. The compound is characterized by:

- 5 (a1): an uncharged morpholino backbone;
- (a2): a base-sequence length of between 12 and 25 nucleotide bases; and
- (a3): a base sequence that is complementary to a target region of a selected preprocessed mRNA coding for a selected protein, where the 5' end of the target region is 1-25 bases downstream of a normal splice acceptor site in the preprocessed mRNA,
- 10 and having the properties that:
 - (b1): the compound is taken up by eukaryotic cells;
 - (b2): the compound hybridizes to the target region of preprocessed mRNA in such cells, and
 - (b3): the compound so hybridized to the target pre-mRNA prevents splicing at the
- 15 normal acceptor splice site, such that the splice mechanism proceeds to a downstream splice acceptor site in the preprocessed mRNA, producing a splice variant processed mRNA with a truncated coding sequence.

In more specific embodiments, the 5' end of the target region is 2-20 bases, or 2-15 bases, downstream of the normal splice acceptor site. The length of the targeting compound is preferably about 15 to 20 nucleotide bases.

In one embodiment, the compound has intersubunit linkages selected from the group consisting of the structures presented in Figs. 2AA-2EE. In preferred embodiments, the linkages are selected from a phosphorodiamidate linkage as represented at Figure 2B-B, where $X=NH_2$, NHR , or NRR' , $Y=O$, and $Z=O$, and an alternate phosphorodiamidate linkage as represented at Figure 2B-B, where $X=OR$, $Y=NH$ or NR , and $Z=O$. R and R' are groups which do not interfere with target binding. Preferably, R and R' are independently selected from alkyl and polyalkyleneoxy (e.g. PEG; $(CH_2CH_2O)_n$), or a combination thereof. The alkyl/polyalkyleneoxy chain may be substituted, preferably at the distal terminus, by a group selected from hydroxy, alkoxy, amino, alkylamino, thiol, 25 alkanethiol, halogen, oxo, carboxylic acid, carboxylic ester, and inorganic ester (e.g. phosphate or sulfonate). Preferably, the chain (independent of substituents) is from 1 to 12 atoms long, and more preferably is from 1 to 6 atoms long. In selected embodiments, R and R' are independently methyl or ethyl. In one embodiment, $X=N(CH_3)_2$, $Y=O$, and $Z=O$.

NRR' may also represent a nitrogen heterocycle having 5-7 ring atoms selected from nitrogen, carbon, oxygen, and sulfur, and having at least as many carbon ring atoms as non-carbon ring atoms. Examples include morpholine, pyrrolidine, piperidine, pyridine, pyrimidine, pyrazine, triazine, triazole, pyrazole, pyrrole, isopyrrole, imidazole, oxazole, imidazole, isoxazole, and the like.

When the downstream splice acceptor site is a whole multiple of three bases downstream of the normal splice acceptor site, the splice variant mRNA has a coding sequence in frame with that of the processed mRNA when it is normally spliced.

The protein is preferably selected from the group consisting of *myc*, *myb*, *rel*, *fos*, *jun*, *abl*, *bcl*, p53, an integrin, a cathedrin, a telomerase, hCG, a receptor protein, a cytokine, a kinase, HIV rev, human papilloma virus, and human parvovirus B19. In selected embodiments, the protein is selected from *myc*, *myb*, *abl*, p53, hCG- β subunit, androgen receptor protein, and HIV-1 rev.

In further selected embodiments, the selected protein has multiple distinct binding regions, as in most transcription factors, and the truncated coding sequence codes for a variant protein in which one such binding region is disabled. Preferably, the variant protein is a dominant negative protein. One example is human *c-myc*, where the variant protein is an N-terminal truncated *c-myc*. In this embodiment, the antisense compound employed has a base sequence selected from the group consisting of SEQ ID NOs: 16 through 32 herein. The variant protein may also be a C-terminal altered *c-myc*, in which case the antisense compound employed can be an 18- to 20-mer having a base sequence which is a contiguous sequence selected from SEQ ID NO: 34; e.g. SEQ ID NO: 33.

In additional exemplary embodiments, the selected protein and the corresponding antisense base sequence(s) targeting its pre-mRNA are selected from the group consisting of:

- (a) human chorionic gonadotropin, β subunit: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 15; e.g. SEQ ID NO: 14;
- (b) human androgen receptor: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 9 or SEQ ID NO: 13; e.g. SEQ ID NO: 8 or 12, respectively;
- (c) human p53: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 36; e.g. SEQ ID NO: 35;
- (d) human *abl*: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 38; e.g. SEQ ID NO: 37; and

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(e) HIV-1 rev: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 41; e.g. SEQ ID NO: 40.

The present invention further provides an antisense compound composed of morpholino subunits joined by phosphorous containing intersubunit linkages and a base sequence between 12 and 25 nucleotide bases in length which is complementary to a target region of a preprocessed mRNA having multiple exons that encode multiple domains in a protein selected from the group consisting of human abl, human p53, human androgen receptor protein, human chorionic gonadotropin (hCG), and HIV-1 rev,

wherein the 5' end of the target region for the antisense compound is 1-25 bases downstream of a normal splice acceptor site within a selected exon in the preprocessed mRNA and the compound alters splicing of the selected protein's preprocessed mRNA to form a variant mRNA encoding a variant protein with altered activity.

The present invention further provides use of an antisense compound composed of morpholino subunits joined by phosphorous-containing backbone linkages and a base sequence between 12 and 25 nucleotide bases in length which is complementary to a target region of a selected preprocessed mRNA having multiple exons that encode multiple domains in a selected protein, wherein the 5' end of the target region for the antisense compound is 1-25 bases downstream of a normal splice acceptor site within a selected exon in the preprocessed mRNA and the compound alters splicing of the protein's preprocessed mRNA to generate a variant mRNA encoding a variant form of the protein with altered activity,

for preparation of a medicament for use in producing, in eukaryotic cells, the variant protein with altered activity for treating a disease affected by the selected protein.

The present invention further provides a method of producing in a eukaryotic cell a variant protein with altered activity for treating a disease affected by the protein, the method comprising administering to the subject an antisense compound composed of morpholino subunits joined by phosphorus-containing backbone linkages and a base sequence between 12 and 25 nucleotide bases in length which is complementary to a target region of a selected pre-processed mRNA having multiple exons and that encode multiple domains in a selected protein, wherein the 5' end of the target region for the antisense compound is 1-25 bases downstream of a normal splice acceptor site within a selected exon in the pre-processed mRNA and the compound alters splicing of the

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proteins pre-processed mRNA to generate a variant mRNA encoding a variant form of the protein with altered activity.

These and other features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Brief Description of the Drawings

Figure 1 shows several preferred subunits having 5-atom (A), six-atom (B) and seven-atom (C-E) linking groups suitable for forming polymers; and

Figures 2A-A to 2E-E show the repeating subunit segment of exemplary morpholino oligonucleotides, designated A-A through E-E, constructed using subunits A-E, respectively, of Figure 1.

Detailed Description of the Invention

I. Definitions

The terms below, as used herein, have the following meanings, unless indicated otherwise:

"Antisense" refers to an oligomer having a sequence of nucleotide bases and a subunit-to-subunit backbone that allows the antisense oligomer to hybridize to a target sequence in an RNA by Watson-Crick base pairing, to form an RNA:oligomer heteroduplex within the target sequence, typically with an mRNA. The oligomer may have exact sequence complementarity to the target sequence or near complementarity. These antisense oligomers may block or inhibit translation of the mRNA, and/or modify the processing of an mRNA to produce a splice variant of the mRNA.

As used herein, the terms "compound", "agent", "oligomer" and "oligonucleotide" may be used interchangeably with respect to the antisense oligonucleotides of the invention.

As used herein, a "morpholino oligomer" refers to a polymeric molecule having a backbone which supports bases capable of hydrogen bonding to typical

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polynucleotides, wherein the polymer lacks a pentose sugar backbone moiety, and more specifically a ribose backbone linked by phosphodiester bonds which is typical of nucleotides and nucleosides, but instead contains a ring nitrogen with coupling through the ring nitrogen. Exemplary structures for antisense oligonucleotides for use in the invention include the morpholino subunit types shown in Figures 1A-E, with the linkages shown in Figures 2A-

4B

A to 2E-E. Such structures are described, for example, in Hudziak *et al.*, *Antisense Nucleic Acid Drug Dev.* 6, 267-272 (1996) and Summerton and Weller, *Antisense Nucleic Acid Drug Dev.* 7, 187-195 (1997).

Subunit A in Figure 1 contains a 1-atom phosphorous-containing linkage which forms the five atom repeating-unit backbone shown at A-A in Figure 2, where the morpholino rings are linked by a 1-atom phosphoamide linkage.

A preferred morpholino oligonucleotide is composed of morpholino subunit structures of the form shown in Fig. 2B-B, where the structures are linked together by phosphorodiamidate linkages, joining the morpholino nitrogen of one subunit to the 5' exocyclic carbon of an adjacent subunit, and P_i and P_j are purine or pyrimidine base-pairing moieties effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. In preferred embodiments, the linkages are selected from a phosphorodiamidate linkage as represented at Figure 2B-B, where X=NH₂, NHR, or NRR', Y=O, and Z=O, and an alternate phosphorodiamidate linkage as represented at Figure 2B-B, where X=OR, Y=NH or NR, and Z=O. R and R' are groups which do not interfere with target binding. Preferably, R and R' are independently selected from alkyl and polyalkyleneoxy (e.g. PEG; (CH₂CH₂O)_n), or a combination thereof. (An example of such a combination would be -(CH₂)₃(CH₂CH₂O)₃-). The alkyl/polyalkyleneoxy chain may be substituted, preferably at the distal terminus (i.e. the terminus not connected to the oligomer backbone), by a group selected from hydroxy, alkoxy, amino, alkylamino, thiol, alkanethiol, halogen, oxo, carboxylic acid, carboxylic ester, and inorganic ester (e.g. phosphate or sulfonate). Preferably, the chain (independent of substituents) is from 1 to 12 atoms long, and more preferably is from 1 to 6 atoms long. In selected embodiments, R and R' are independently methyl or ethyl. In one embodiment, X=N(CH₃)₂, Y=O, and Z=O. NRR' may also represent a nitrogen heterocycle having 5-7 ring atoms selected from nitrogen, carbon, oxygen, and sulfur, and having at least as many carbon ring atoms as non-carbon ring atoms. Examples include morpholine, pyrrolidine, piperidine, and pyridine.

Subunits C-E in Figure 1 are designed for 7-atom unit-length backbones as shown for C-C through E-E in Figure 2. In Structure C, the X moiety is as in Structure B and the moiety Y may be a methylene, sulfur, or preferably oxygen. In Structure D the X and Y moieties are as in Structure B. In Structure E, X is as in Structure B and Y is O, S, or NR. In all subunits depicted in Figures 1A-E, Z is O or S, and P_i or P_j is adenine, cytosine, guanine or uracil.

A "nuclease-resistant" oligomeric molecule (oligomer) is one whose backbone is not susceptible to nuclease cleavage.

As used herein, an oligonucleotide or antisense oligomer "specifically hybridizes" to a target polynucleotide if the oligomer hybridizes to the target under physiological
5 conditions, with a T_m substantially greater than 37°C, preferably at least 50°C, and typically 60°C-80°C or higher. Such hybridization preferably corresponds to stringent hybridization conditions, selected to be about 10°C, and preferably about 50°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. At a given ionic strength and pH, the T_m is the temperature at which 50% of a
10 target sequence hybridizes to a complementary polynucleotide.

Polynucleotides are described as "complementary" to one another when hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides. A double-stranded polynucleotide can be "complementary" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the
15 second. Complementarity (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonds with each other, according to generally accepted base-pairing rules.

A "RNase-inactive" or "RNase-incompetent" oligonucleotide or oligonucleotide
20 analog is one which acts via an RNase-independent mechanism, unlike RNase-active oligonucleotides, such as phosphorothioates. They are believed to function by sterically blocking target RNA formation, nucleocytoplasmic transport or translation, and are thus also referred to as "steric blockers". This class includes, for example, methylphosphonates, morpholino oligonucleotides, as described herein, peptide nucleic acids (PNA's), and 2'-O-allyl or 2'-O-alkyl modified oligonucleotides.
25

In a "peptide nucleic acid", the deoxyribose phosphate units of an oligonucleotide backbone are replaced with polyamide linkages. Proper backbone spacing is attained by the use of 2-aminoethyl glycine units, with a nucleotide base attached to each 2-amino group via a methylenecarbonyl group.

30 A "2'-O-allyl (or alkyl) modified oligonucleotide" is an oligoribonucleotide in which the 2' hydroxyl is converted to an allyl or alkyl ether. The alkyl ether is typically a methyl ether.

"Alkyl" refers to a fully saturated acyclic monovalent radical containing carbon and

hydrogen, which may be branched or a straight chain. Examples of alkyl groups are methyl, ethyl, n-butyl, t-butyl, n-heptyl, and isopropyl. "Lower alkyl" refers to an alkyl radical of one to six carbon atoms, and preferably one to four carbon atoms, as exemplified by methyl, ethyl, isopropyl, n-butyl, isobutyl, and t-butyl.

5 A "truncated" protein or coding sequence has some portion of the normal protein or sequence removed from one or the other terminus, from an internal region, or a combination of the above.

An amino-truncated (N-truncated) or carboxy-truncated (C-truncated) protein is one having an abnormal or deleted amine terminus or carboxy terminus, respectively, arising
10 from translation of a splice variant mRNA.

II. Antisense Compounds

In accordance with the present invention, it has been discovered that an antisense compound having from 12 to 25 nucleotides, including a targeting base sequence that is
15 complementary to a target region of a selected preprocessed mRNA coding for a selected protein, where the 5' end of the target region is 1 to 25 bases downstream, preferably 2 to 20 bases downstream, and more preferably 2 to 15 bases downstream, of a normal splice acceptor site in the preprocessed mRNA, is effective to inhibit splicing at the normal splice acceptor site and thus produce splice variant mRNA, leading to truncated or
20 otherwise aberrant versions of the selected protein upon translation. Advantages of this strategy are set forth below.

The antisense compound employed in the present invention is one that does not activate RNase H. RNase-H active oligomers, of which phosphorothioate oligonucleotides are the most prominent example, operate primarily by a mechanism in
25 which the target mRNA is cleaved. RNase-incompetent oligomers, on the other hand, are believed to act by a steric blocking mechanism. Such compounds include morpholino oligomers, PNA's (peptide nucleic acids), methylphosphonates, and 2'-O-alkyl or -allyl modified oligonucleotides, all of which are known in the art. The preferred antisense oligomers (compounds) of the present invention are morpholino oligomers, which are
30 composed of morpholino subunits of the form shown in U.S. Patent Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,521,063, and 5,506,337, all of which are incorporated herein by reference. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in these patents. In a morpholino oligomer, (i) the morpholino groups are linked together by uncharged phosphorus-containing linkages, one

to three atoms long, joining the morpholino nitrogen of one subunit to the 5' exocyclic carbon of an adjacent subunit, and (ii) the base attached to the morpholino group is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. The purine or pyrimidine base-pairing moiety is typically adenine, cytosine, guanine, uracil or thymine. Preparation of such oligomers is described in detail in U.S. Patent No. 5,185,444 (Summerton and Weller, 1993), which is hereby incorporated by reference in its entirety. As shown in the reference, several types of nonionic linkages may be used to construct a morpholino backbone.

Such morpholino oligomers have shown high binding affinity for RNA targets, and the uncharged backbone favors uptake into cells and reduces non-specific binding interactions, relative to charged analogs such as phosphorothioates. They have been shown to provide significantly improved activity and selectivity in inhibiting translation of targeted sequences in comparison to phosphorothioate oligonucleotides. See, for example, Summerton *et al.*, *Antisense & Nucleic Acid Drug Dev.* 7(2):63-70, Apr 1997. The morpholino oligomers have very high nuclease resistance and good water solubility, making them good candidates for *in vivo* use. Efficient uptake by cells *in vivo* is demonstrated in co-owned and copending application serial no. 09/493,427 and the corresponding PCT Pubn. No. WO 0044897. As described therein, morpholino oligonucleotides having phosphoramidate linkages formed heteroduplexes with target RNA, which are protected in this duplex state from nuclease degradation. Such a duplex is expelled from the cell, and the target RNA can later be detected in a body fluid sample from the subject. These results demonstrated that the morpholino oligomers (i) migrate to and enter cells in the body and (ii) bind with high affinity, via Watson-Crick base-pairing, to target nucleic acid regions.

Exemplary backbone structures for antisense oligonucleotides of the invention include the β -morpholino subunit types shown in Fig 1A-E, as described above. It will be appreciated that a polynucleotide may contain more than one linkage type.

A preferred morpholino oligonucleotide is composed of morpholino subunit structures of the form shown in Fig. 2B-B, where the structures are linked together by phosphorodiamidate linkages, joining the morpholino nitrogen of one subunit to the 5' exocyclic carbon of an adjacent subunit, P_i and P_j are purine or pyrimidine base-pairing moieties effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. In preferred embodiments, the linkages are selected from a

phosphorodiamidate linkage as represented at Figure 2B-B, where $X=NH_2$, NHR , or NRR' , $Y=O$, and $Z=O$, and an alternate phosphorodiamidate linkage as represented at Figure 2B-B, where $X=OR$, $Y=NH$ or NR , and $Z=O$. R and R' are groups which do not interfere with target binding. Preferably, R and R' are independently selected from alkyl
5 and polyalkyleneoxy (e.g. PEG; $(CH_2CH_2O)_n$), or a combination thereof. The alkyl/polyalkyleneoxy chain may be substituted, preferably at the distal terminus, by a group selected from hydroxy, alkoxy, amino, alkylamino, thiol, alkanethiol, halogen, oxo, carboxylic acid, carboxylic ester, and inorganic ester (e.g. phosphate or sulfonate). Preferably, the chain (independent of substituents) is from 1 to 12 atoms long, and more
10 preferably is from 1 to 6 atoms long. In selected embodiments, R and R' are independently methyl or ethyl. In one embodiment, $X=N(CH_3)_2$, $Y=O$, and $Z=O$. NRR' may also represent a nitrogen heterocycle having 5-7 ring atoms selected from nitrogen, carbon, oxygen, and sulfur, and having at least as many carbon ring atoms as non-carbon ring atoms. Examples include morpholine, pyrrolidine, piperidine, pyridine, pyrimidine,
15 pyrazine, triazine, triazole, pyrazole, pyrrole, isopyrrole, imidazole, oxazole, imidazole, isoxazole, and the like.

The solubility of the antisense compound, and the ability of the compound to resist precipitation on storage in solution, can be further enhanced by derivatizing the oligomer with a solubilizing moiety, such as a hydrophilic oligomer, or a charged moiety, such as a
20 charged amino acid or organic acid. The moiety may be any biocompatible hydrophilic or charged moiety that can be coupled to the antisense compound and that does not interfere with compound binding to the target sequence. The moiety can be chemically attached to the antisense compound, e.g., at its 5' end, by well-known derivatization methods. One preferred moiety is a defined length oligo ethylene glycol moiety, such as
25 triethyleneglycol, coupled covalently to the 5' end of the antisense compound through a carbonate linkage, via a piperazine linking group forming a carbamate linkage with triethyleneglycol, where the second piperazine nitrogen is coupled to the 5'-end phosphorodiamidate linkage of the antisense. Alternatively, or in addition, the compound may be designed to include one a small number of charged backbone linkages, such as a
30 phosphodiester linkage, preferably near one of the ends of the compound. The added moiety is preferably effective to enhance solubility of the compound to at least about 30 mgs/ml, preferably at least 50 mgs/ml in aqueous medium.

The compound is designed to hybridize to the target sequence under physiological

conditions with a T_m substantially greater than 37°C, e.g., at least 50°C and preferably 60°C-80°C. Although the compound is not necessarily 100% complementary to the target sequence, it is effective to stably and specifically bind to the target sequence such that expression of the target sequence, is modulated. The appropriate length of the oligomer to allow stable, effective binding combined with good specificity is about 8 to 40 nucleotide base units, and preferably about 12-25 base units. Mismatches, if present, are less destabilizing toward the end regions of the hybrid duplex than in the middle. Oligomer bases that allow degenerate base pairing with target bases are also contemplated, assuming base-pair specificity with the target is maintained.

III. Selection of Target Sequences

A. RNA Splicing: Background

The processing of nuclear RNA following transcription is observed in virtually all living cells. The mammalian genome contains genes that make transcripts of approximately 16,000 bases in length containing 7 to 8 exons. The process of splicing reduces the length of the mRNA to an average of 2,200 bases. The initial transcript is referred to as heterologous nuclear RNA (hnRNA) or pre-mRNA. Processing of hnRNA involves an aggregate of approximately 20 proteins, referred to collectively as the spliceosome, which carries out splicing and transport of mRNA from the nucleus. The spliceosome does not appear to scan from a common direction for all transcripts; introns may be removed in a reproducible order but not in a directional order. For example, introns 3 and 4 may be removed first, followed by removal of introns 2 and 5, followed by removal of introns 1 and 6. The order of intron removal is not predictable a priori of observation. The sequence recognition for processing is small, suggesting that errors or multiplicity of processing sites can be anticipated, and, in fact, as more genes are investigated, more variation in processing of hnRNA has been observed.

In preprocessed mRNA, the two-base sequence motifs at exon/intron junctions are invariant. The upstream (5') splice donor (SD) junction is of the form exon-/GT-intron, while the downstream (3') splice acceptor (SA) junction is of the form intron-AG/exon. The flanking bases are not invariant; however, the base immediately upstream of the splice acceptor AG sequence is C about 80% of the time.

The current understanding of intron sequence recognition is as follows:

5'-exonA₍₆₄₎G₍₇₃₎ /G₍₁₀₀₎T₍₁₀₀₎A₍₆₂₎A₍₆₈₎G₍₈₄₎T₍₆₃₎ ...TACTAAC...C₍₈₀₎ A₍₁₀₀₎G₍₁₀₀₎ / exon-NN...-3'

splice donor (SD)

splice acceptor (SA)

The numbers in parenthesis represent the approximate (when <100%) percent utilization of a base at a site. The bold A in the middle of the intron is the site (branch point) at which the G from the splice donor forms a branched 2'-5'-structure referred to as the lariat. The sequence indicated (TACTAAC) is the consensus observed in yeast. The consensus sequence in mammalian cells is PyNP₍₈₀₎Py₍₈₇₎Pu₍₇₅₎APy₍₇₅₎, where Py represents a pyrimidine (U/T or C) and Pu represents a purine (A or G). The A at the branch point is invariant, and is typically found about 12 to 50 bases upstream of the AG site. A pyrimidine-rich region (not shown above) is also generally found near the 3' end of the intron, about 10-15 bases upstream of the AG site.

Once the spliceosome forms the lariat, two transesterification reactions take place: 1) the 2'-OH of the branch point A in the intron to the 5'-phosphate of the SD intron G, and 2) the 3'-OH of the SD exon G to the 5'-phosphate of the first base of the SA exon. The removed intron is rapidly degraded in most cases, and the joined exons are now referred to as mature mRNA, which is transported out of the nucleus for translation into proteins by the ribosome.

20 B. Targeting Strategies

Various approaches could be taken to targeting the processing (splicing) of mRNA by antisense oligomers. The following sites could be targeted:

1. The exon-only portion of the SD exon (upstream of SD site), to interfere with SD processing.
- 25 2. The SD exon/intron junction, to interfere with SD processing.
3. The site of lariat formation within the intron, to block spliceosomal recognition upstream of the SA exon.
4. The SA intron/exon junction, to interfere with SA processing.
5. The exon-only portion of the SA exon (downstream of SA site), to interfere with SA processing.
- 30

Prior art methods have utilized strategy 2 or 4 (targeting of SD or SA junction). See, for example, RV Giles *et al.*, cited above, in which a morpholino antisense oligomer spanning a splice acceptor site in the *c-myc* gene is described.

35 Experiments in support of the present invention found that targeting strategies 4 and 5

above, directed to the splice acceptor, were more reliably effective than strategies 1 and 2, directed to the splice donor.

For example, studies were carried out with rat CYP3A2 pre-mRNA targeted *in vivo* (whole animal). Animals were injected i.p. with 100 µg PMO (as shown in Fig. 2B-B, where Y₁ and Z are oxygen and X is N(CH₃)₂) in phosphate buffered saline. The diminished rate of microsomal metabolism of erythromycin O-demethylase was monitored to reflect the expected phenotype caused by the antisense inhibition. As shown, the splice donor (SD) targeting was less effective than the splice acceptor (SA) strategy:

10	ANTI-SENSE			
	<u>STRATEGY</u>	<u>SEQUENCE (/ indicates splice junction)</u>	<u>SEQ ID NO:</u>	<u>ERDEM % of control</u>
	Control	Saline	—	100 ± 10.2 (N=7)
	SD	3'-AAGAGATGGC/CACTCACTGG-5'	4	94.7 ± 5.4 (N=3)
	SA	3'-GGAAATATC/TGAACCTTGGG-5'	5	86.5 ± 3.8 (N=3)

Experiments with oligomers antisense to *c-myc* mRNA were conducted in cultured rat NRK cells, evaluating the inhibition of cellular DNA synthesis by monitoring incorporation of tritiated thymidine. Sequences were derived from Genbank Acc. No. Y00396 (rat) and J00120 (human), targeting the splice acceptor region at the beginning of exon 2 (with the exception of SEQ ID NO: 1, which targets the splice donor). The rat and human sequences indicated are highly homologous in this region. The oligomers listed in Table 1, below, were screened for antiproliferative effects using several proliferation assays. Primary fibroblasts from two species, rat and human, NRK and WI-38, respectively, were used for the screening. Data shown in Table 1 employed NRK cells.

Data from [³H]thymidine incorporation assays using 20 µM PMO (as shown in Fig. 2B-B, where Y₁ and Z are oxygen and X is N(CH₃)₂) are presented in Table 1. Percentages refer to [³H]thymidine incorporation relative to the vehicle (H₂O) control. Therefore, the lower the number, the greater the antiproliferative effect. It can be seen that all oligomers tested exhibited at least some antiproliferative activity. The extent of the inhibitory activity compared favorably with the antiproliferative drug Taxol (Paclitaxel, Bristol-Myers Squibb, Princeton, NJ) at 32% of control. As 10%-20% of the cells are not affected by the scrape loading procedure and will, therefore, contribute to the residual [³H]thymidine incorporation activity, it is likely that most or all cells containing the most efficacious oligomers were growth inhibited.

TABLE 1. Cell Growth Inhibition by Anti-*c-myc* and Control Sequences

SEQ ID NO:	Antisense Sequence (5'→3')	Targeted Region*	Incorporation vs. vehicle control
1	CTGTGCTTAC/CGGGTTTTCCACCTCCC (/ = SD site)	2553-2579	51 ± 8%
2	ATCGTCGTGACTGT/CTGTTGGAGGG (/ = SA site)	4140-4164	27 ± 3%
3	GCTCACGTTGAGGGGCATCG	4161-4180	38 ± 2%
25	ACGTTGAGGGGCATCGTCGC	J00120 4515-34	29%
42	GGGGCAUCGUCGUGACUGU/CUGUUGGAGG	4140-4169	20%
43	CGUCGUGACUGU/CUGUUGGAGG	4141-4162	45%
44	CGTCGTGACTGT/CTGTTGGAGG	4141-4162	21%
45	GGCAUCGUCGCGGGAGGCUG/CUGGAGCG	J00120 4498-4505	22%
46	CCGCGACAUAGGACGGAGAGCAGAGCCC	4364-4391	56%
47	ACTGTGAGGGCGATCGCTGC (scrambled)	--	~100%
48	ACGATGAGTGGCATAGTCGC (3 mismatches)	--	>100%
49	CTCCGCAATGCTGAAAGGTG (rat BCL-2)	--	>100%
50	GGCGUGCCUCAACAUGGUGGCGG (rat PCNA-1)	--	~100%

* Genbank Y00396 (rat) unless otherwise indicated

- Two irrelevant-sequence oligomers (rat BCL-2, SEQ ID NO: 49 and rat PCNA-1, SEQ ID NO: 50) did not inhibit NRK cells at the highest concentration assayed (20 μ M). Mismatched and scrambled sequences of SEQ ID NO: 25 (SEQ ID NOs 48 and 47, respectively) had no effect on the proliferation of WI-38 (human) fibroblasts at 20 μ M.

- SEQ ID NO: 45, which spans the 3'-splice acceptor site of the first intron of human *myc* pre-mRNA, was shown by Giles *et al.* (1999) to cause missplicing of *myc* pre-mRNA. A cryptic or latent splice acceptor, 44 bp distal to the normal splice acceptor position, was used to produce an aberrantly spliced mRNA with a 44-bp deletion. This misspliced mRNA lacks the initiator AUG and did not produce a normal *myc* protein.

SEQ ID NO: 25 does not span the intron 1-exon 2 boundary, the first potential complementary base pair being the tenth nucleotide from the 3' splice acceptor. Similarly,

SEQ ID NO: 3 has its 5' end eleven bases downstream of the splice acceptor in the rat *c-myc* mRNA sequence. As the data shows, both sequences were effective in inhibiting cell growth. It was of interest to determine if these oligomers inhibited normal splicing, as had been shown for SEQ ID NO: 45. Accordingly, RNA was prepared from human cells
5 treated with positive (SEQ ID NO: 45) and negative (SEQ ID NO: 50) control oligomers as well as SEQ ID NO: 25. The structure of the RNA was then analyzed by making a DNA copy with reverse transcriptase and then performing PCR with flanking DNA primers (see Materials and Methods, below). The products of the RT-PCR procedure were analyzed by agarose gel electrophoresis.

10 In untreated or irrelevant oligomer-treated cells, a 304-bp band is predicted, and was observed, from the primers used and the *c-myc* nucleotide sequence. For cells treated with SEQ ID NO: 25, two DNA bands were observed. The upper band, which comigrated with the fragment from untreated or negative control cells, represented mRNA from the 10%-20% of cells not scraped loaded by the uptake procedure used, plus the fraction of correctly
15 spliced mRNA in treated cells. The heavier lower band represented the misspliced, 44-bp depleted mRNA.

Cells loaded with SEQ ID NO: 25 also produced two bands from the RT-PCR procedure, one the size of normally spliced mRNA and one smaller. It can therefore be concluded that SEQ ID NO: 25, despite not directly overlapping the splice acceptor site, is
20 capable of causing missplicing. The irrelevant PMO (SEQ ID NO: 50) gave an mRNA structure pattern identical to that of untreated cells, demonstrating that the missplicing is sequence specific.

A dose-response study with SEQ ID NO: 25 gave an IC_{50} of 3 μ M. The inhibitory effects began to plateau at 10 μ M, and there was little further change from 10 μ M to 20
25 μ M.

Myc protein has been implicated as important for the transition from G_0/G_1 of the cell cycle into the S phase (MK Mateyak *et al.*, *Cell Growth Differ.* 8:1039-48, 1997). It could, therefore, be expected that if *myc* protein levels are reduced, the cells would arrest in G_1 . This effect was investigated by determining the number of cells in G_1 and G_2 , using
30 the method of Telford *et al.* (*Cytometry* 13:137-43, 1993) (see Materials and Methods). After removal of RNA by hydrolysis, cells were stained by propidium iodide, a DNA-specific fluorescent dye. The DNA content per cell distribution was then determined by

FACS analysis. The FACS intensity profiles show two peaks, corresponding to 2N DNA content (G₁) and 4N DNA (G₂). Cells treated with the PMO having SEQ ID NO: 25 showed an increase in the proportion of cells in G₁ (79% compared to 66% vehicle control) compared with those in G₂ (9% compared to 21% vehicle control). A positive control of quiescent cells obtained by growth factor starvation showed 80% of cells in G₁ and 8% in G₂.

To obtain further evidence that the anti-*c-myc* oligomer SEQ ID NO: 25 inhibits *c-myc* mRNA expression by an antisense mechanism, a reporter gene model system was constructed to directly examine one gene (luciferase) and its activity (light production).

The *myc*-luciferase reporter plasmid contained the entire 2.2-kb 5' region of human *c-myc* and the first 6 amino acid codons of the *myc* protein fused to the insect luciferase cDNA (see Hudziak *et al.*, *Antisense Nucleic Acid Drug Dev.* 10:163-76 (2000). It was transfected into HeLa cells, and a luciferase-producing clone was selected and designated clone L6. This cell line was scrape loaded with the indicated concentrations of PMO and replated. After 24-30 hours, the cells were lysed, and luciferase activity was measured (see Materials and Methods).

The results showed strong inhibition of luciferase production, with an IC₅₀ of 300 nM. Several control experiments were performed to determine if the observed inhibition of luciferase synthesis was due to sequence-specific inhibition by the PMO. SEQ ID NO: 25 and the two sequence permutations noted above (SEQ ID NOs: 47 and 48) were compared. Neither the scrambled version nor the 3-base mispair oligomer had any effect on luciferase production. Other control experiments showed that SEQ ID NO: 25 had no effect on HeLa proliferation during the 30-hour incubation time of the luciferase assay, and had no effect on an unrelated target reporter system (rabbit α -globin; J Summerton *et al.*, *Antisense & Nucleic Acid Drug Dev.* 7:63-70, 1997) under conditions where a sequence complementary agent gave 70% inhibition. Other sequences containing 3-4 contiguous guanine (G) bases were tested in the NRK cell proliferation assay described above, and gave no significant inhibition of cell proliferation at concentrations up to 20 μ M.

In an evaluation of the "functional footprint" of PMO antisense directed to the splice acceptor (SA) region of rat *c-myc*, interference in splicing was observed with PMOs targeting the region from -44 upstream of the SA to +36 downstream (3' end of target region) from the SA. As the target sequence moved downstream, the proportion of lower

molecular weight proteins, relative to normal *c-myc*, was observed to increase.

Targeting downstream of the splice acceptor junction, i.e. within the exon, is generally preferred, for reasons discussed below.

5 IV. Consequences of Interference with SA Site

If a binding oligomer interferes with normal mRNA splicing at the SA site, the spliceosome will proceed to the next best unblocked candidate site in the region. This site tends to be a [C]AG sequence with a short run of pyrimidines 10 to 15 bases upstream and a suitable branch point further upstream.

10 The resulting processed mRNA will generally be a splice variant mRNA in which the sequence between the normal splice acceptor site and the alternate (or "cryptic") splice acceptor site has been deleted. The resulting variant protein can take different forms depending on the deleted sequence. For example, if the deleted sequence contains a whole multiple of three base pairs (that is, the downstream splice acceptor site is a whole
15 multiple of three bases downstream of the normal splice acceptor site), the subsequent sequence will be in frame with the normal sequence, and a truncated form of the native protein will result. This permits the formation of dominant negative proteins, as described, for example, in Example C below. If the cryptic site is out of frame with the normal site, however, an unrelated "nonsense" protein will be produced. Due to the
20 frequent occurrence of stop codons in non-reading frame sequences (about one per twenty codons), such a splice variant mRNA typically results in early termination of translation.

If the deleted sequence includes an AUG start site, translation may then occur at an alternate AUG site found further downstream. Again, if this alternate site is in frame with the normal AUG site, the resulting protein will have a deletion of some number of amino
25 acids from the native protein. If the alternate site is out of frame, a "nonsense" protein, typically truncated early in translation, will result.

In one embodiment, exemplified in Examples A and B below, a carboxy-terminal truncated protein is created. In another embodiment, exemplified in Examples C and D below, a protein having an abnormal or deleted amino terminus is produced. The latter
30 can be accomplished if the AUG translation start site is in exon 2 or greater of the mRNA.

Thus, when the sequence of pre-mRNA for a desired protein is known, an antisense target can be designed to alter desired regions of the protein. Preferably, the binding domains of the protein are also known, such that selected functions of the protein can be altered.

The following steps can be used to target genes with multiple exons, in accordance with the invention:

- Step 1. Identify functional domains of the protein in question. The scientific literature can provide most of this information. One excellent source is "The Oncogene FactsBook" by Robin Hesketh, Academic Press, London, 1995, in which proteins related to cancer are reviewed and their functional domains mapped. Similarly, the "Cytokine FactsBook" (RE Callard and AJH Gearing, Academic Press) describes functional domains of cytokines. Other works in the same series include "The Protein Kinase Factsbook", "The G-Protein Linked Receptor Factsbook", and "The Extracellular Matrix Factsbook".
- Step 2. Search GenBank or similar nucleic acid databases for the gene sequence, including exon/intron sequences and junctions.
- Step 3. Preferred antisense targets are within approximately 35-40 downstream bases of a splice acceptor site. Preferably, the target region has its 5' end at a location 1 to 25 bases downstream, more preferably 2 to 20 bases downstream, and most preferably 2 to 15 bases downstream, from the SA junction. (Note that a sequence one base downstream directly abuts, but does not overlap, the [C]AG splice acceptor site, and includes the first base of the normal exon.)
- Step 4. To predict the consequence of inhibition, search the exon downstream of the SA for a cryptic splice acceptor, i.e. a [C]AG sequence downstream from the authentic SA. Once such a candidate site is located, search for a 5'-TACTAAC-3' (or similar) site for lariat formation 12 to 50 bases upstream of the cryptic SA. (Note that, according to the consensus sequence given above, only the branch point A must be conserved, so there is considerable flexibility in this sequence.) Preferably, there should also be a short pyrimidine-rich region 10-15 bases upstream of the cryptic SA.
- Step 5. Once a potential cryptic SA site is identified, determine if its use will result in "in frame reading" by dividing the number of bases between the authentic site and the cryptic site by 3. If the quotient is a whole number, then the resultant protein will be "in frame" and possibly a dominant negative protein (see below).

Accordingly, in one preferred embodiment, an antisense target is chosen such that splicing is likely to be directed to a cryptic site whose use will result in in-frame reading.

V. Target Proteins and Selected Examples

Suitable target proteins include, for example, transcription factors, particularly oncogenic or proto-oncogenic proteins such as *myc*, *myb*, *rel*, *fos*, *jun*, *abl*, *bcl*, and *p53*; matrix proteins, such as integrins and cathedrins; other tumor-expressed proteins, such as hCG; telomerases; receptor proteins; cytokines; kinases; and viral proteins, such as HIV rev, human papilloma virus, and human parvovirus B19. It is appreciated that inhibition of such proteins has numerous therapeutic applications. These include, but are not limited to, antitumor therapy, e.g. by targeting proteins, such as transcription factors, involved in various aspects of cell division and cell cycle regulation; antiviral or antibacterial therapy, by targeting proteins essential to replication or other vital functions of the infectious agent; and inhibition of restenosis or other proliferative disorders, by inhibiting proteins which support cell proliferation at the site.

Transcription factors are typically multidomain proteins, having a DNA binding region and a protein-protein binding region. Interfering with one of these regions can produce a dominant negative protein, which counters the activity of the native protein by preserving one activity (such as protein binding) while inhibiting another activity critical to the proper function of the protein (such as DNA binding; or vice versa). See the *c-myc* example described below.

As noted above, functional domains of many of the target proteins noted above have been studied extensively and reported in the literature. Sequences of pre-mRNA, including locations of introns, exons, and AUG start codons, can be found in the GenBank sequence database or other published sources readily available to those of skill in the art.

Following are several examples of antisense targeting downstream of splice acceptor domains in selected proteins to produce splice variants mRNAs which, upon translation, produce proteins with specific alterations.

In one embodiment, exemplified in Examples A and B, a carboxy-terminal truncated protein is created. In another embodiment, exemplified in Examples C and D, a protein having an abnormal or deleted amino terminus is produced.

30 A. Human androgen receptor (GenBank M35845, M35846)

A review of prostate cancer molecular biology indicates that androgen ablation is the state of the art therapy. In accordance with the present invention, this can be accomplished by inactivation of the androgen receptor. By targeting the center of the gene, at exon 2, various options are available for inhibiting expression of a functional

androgen receptor. Proposed targeting of the splice acceptor in exon 2 (SEQ ID NO: 8; GenBank M35845) or exon 3 (SEQ ID NO: 11; GenBank M35846) is indicated.

End of Intron 1: 5'-...TGTGTCTTTTCCAG/ ← splice acceptor site (SEQ ID NO: 6)

5 Exon 2: 5'-TTTGGAGACTGCCAGGGACCATG...-3' (SEQ ID NO: 7)

Target antisense sequence: 5'-CATGGTCCCTGGCAGTCTCC-3' (SEQ ID NO: 8)

This oligomer (SEQ ID NO: 8) targets the sequence starting at base 48, four bases downstream of the normal splice acceptor site. The next probable cryptic splice site is at base 100 (CAG at 97-99; branch point A at 80; upstream pyrimidine region at 87-92. This site is out of frame with the normal site and should thus result in early termination of the protein.

Similar length oligomers targeting sequences having a 5' end 1 to about 18 bases downstream of the splice acceptor could also be used. This includes oligomers having a length of about 18 contiguous nucleotides selected from the sequence 5'-TCA ATG GGC AAA ACA TGG TCC CTG GCA GTC TCC AAA -3' (SEQ ID NO: 9; complementary to bases 45-80 of the sequence given in Genbank Acc. No. M35845).

End of intron 2: 5'-...TTTGTGTTCTCCAG/ ← splice acceptor site (SEQ ID NO: 10)

Exon 3: 5'-GGAAACAGAAGTACCTGTGCGCC...-3' (SEQ ID NO: 11)

20 Antisense sequence: 5'-GGC GCA CAG GTA CTT CTG-3' (SEQ ID NO: 12)

This oligomer (SEQ ID NO: 12) targets the sequence starting at base 49, six bases downstream of the normal splice acceptor site. A possible cryptic splice site is at base 145 (CAG at 143-145; branch point A at 114; pyrimidine region at 123-127). This site is in frame with the normal splice site and should thus result in a carboxy-truncated version of the native protein.

Similar length oligomers targeting sequences having a 5' end 1 to about 18 bases downstream of the splice acceptor could also be used. This includes, for example, oligomers having a length of about 18-20 contiguous nucleotides selected from the sequence 5'- AAT CAT TTC TGC TGG CGC ACA GGT ACT TCT GTT TCC -3' (SEQ ID NO: 13; complementary to bases 44-79 of the sequence given in Genbank Acc. No. M35846).

B. Human chorionic gonadotropin (β subunit) (GenBank X00266)

The β subunit of hCG is nearly identical to leutinizing hormone (LH) with the exception of the COOH end, which is extended in hCG. This extension is in exon 3 of the gene. By interfering with the exon 3 SA site, hCG could be truncated without targeting LH, unlike targeting of the AUG translation initiation sites, which are highly conserved in both proteins.

An oligomer with the sequence 5'-CCC CTG CAG CAC GCG GGT-3' (SEQ ID NO: 14) binds in exon 3 near (directly abutting) the SA (CAG at 1318-1320), targeting the sequence from bases 1321-1338, and interferes with splicing at this site. Similar length oligomers targeting sequences having a 5' end at base 1322, 1323, etc., up to about base 1340, could also be used. This includes, for example, oligomers having a length of about 18-20 contiguous nucleotides selected from the sequence 5'- GAG GCA GGG CCG GCA GGA CCC CCT GCA GCA CGC GGG T-3' (SEQ ID NO: 15; complementary to bases 1321-57 of the sequence given in Genbank Acc. No. X00266).

Possible cryptic SA sites are at base 1393 (AG at 1391-2; branch point A at 1370 or 1373; in frame) and at 1458 (CAG at 1455-7; branch point A at 1427; out of frame). Splicing at either site would delete at least 24 amino acids from the protein, and possibly more, if the out of frame site were used. Since hCG is expressed only in tumor cells, this would be therapeutically beneficial in that less protein is expressed, and the protein will have a shorter biological half-life. In addition, the truncated protein may have unusual amino acids at the COOH end, possibly producing an immune response to hCG, useful in a vaccination strategy.

C. Human *c-myc* (GenBank J00120)

c-myc is a proto-oncogene which regulates cell growth and differentiation and is involved in the processes of vascular remodeling, smooth muscle cell proliferation, extracellular matrix synthesis, and apoptosis. Aberrant expression of *c-myc* is frequently observed in human cancer. Aberrant, constitutive or overexpression of *c-myc* has been associated with a number of human cancers including lung cancer, colorectal cancer, breast cancer, bladder cancer, leukemia, lung cancer, etc. It has also been demonstrated that inhibition of *c-myc* reduces the incidence and severity of restenosis.

The *c-myc* protein has a DNA binding domain in the amine-terminal portion of the sequence and a protein-protein interacting domain in the carboxy-terminal portion. It is known that *c-myc* binds with *Max* in the carboxy domain to form a heterodimer that can

bind to a DNA sequence known as an E-Box (5'-CACGTG-3'). When *myc:max* binds in this manner, the phenotype is growth stimulatory and can lead to apoptosis.

If *myc* concentration is low, then *max* forms homodimers which do not transactivate. If the *mad* protein is induced, then *max* binds *mad* to form *mad:max* heterodimers, which
 5 tend to induce differentiation and are anti-apoptotic.

Hence, simply inhibiting *myc* tends to result in the *mad:max* phenotype, which is anti-apoptotic. However, if the DNA binding (amine-terminal) domain of *c-myc* is inhibited, while leaving the protein-protein binding (carboxy-terminal) domain intact, the result is a dominant negative protein capable of binding *max* but not capable of transactivation. The
 10 favorable phenotype would be the loss of growth stimulatory actions, but the compensatory anti-apoptotic actions of *mad:max* would not dominate, as *max* protein can still bind to the *myc* COOH domain.

Inhibition of the amine-terminal domain is achieved by appropriate design of an antisense compound to direct alteration of splicing. Cryptic (alternate) SA sites found
 15 downstream include:

- 4547 Possible candidate; the next AUG, at 4554, is out of frame and would thus produce unrelated ("nonsense") protein. (Could be blocked by oligos targeted further downstream than SEQ ID NO: 13)
- 20 4578 Not a good SA candidate, due to few pyrimidines upstream
- 4617 A good SA candidate; next downstream AUG is at 4821, which is in frame with normal AUG at 4521

As described above, a morpholino oligomer (PMO) having a sequence
 25 complementary to bases 4515 to 4534 of the human *c-myc* mRNA sequence (Genbank Accession No. J00120), ten bases downstream of the splice acceptor site AG at 4504-5 (SEQ ID NO. 25, 5'-ACG TTG AGG GGC ATC GTC GC-3'), was found to prevent appropriate splicing at this site and the use of the normal AUG translation start site at 4521. Analysis of the mRNA produced indicated use of the 4617 site indicated above.
 30 Translation starting at the AUG at 4821, 300 bases downstream of the normal AUG, produces a protein having a 100 amino acid deletion at the N-terminus (as also reported by Giles *et al.*) Use of antibodies to the COOH end of *myc* revealed this protein, as well as substantially smaller proteins, from cells treated with the antisense oligomer. These N-terminal truncated proteins are expected to bind *max* but will not bind DNA.

35 These results, combined with examination of primer-amplified mRNAs, demonstrated

the use of the 4617 cryptic splice site, as well as other cryptic sites further downstream.

As noted above, PMO oligomers targeted to the region from -44 upstream of the SA to +36 downstream (ds) from the SA showed evidence of interference with splicing in rat *c-myc*. Assuming an oligomer length of about 20 bases, the following antisense sequences

5 could thus be utilized for targeting of human *c-myc*:

Table 2

Antisense Sequence	Bases ds of SA site (5' end of sequence)	SEQ ID NO:
5'-GGCATCGTCGCGGGAGGCTG-3'	1	16
5'-GGGCATCGTCGCGGGAGGCT-3'	2	17
5'-GGGGCATCGTCGCGGGAGGC-3'	3	18
5'-AGGGGCATCGTCGCGGGAGG-3'	4	19
5'-GAGGGGCATCGTCGCGGGAG-3'	5	20
5'-TGAGGGGCATCGTCGCGGGA-3'	6	21
5'-TTGAGGGGCATCGTCGCGGG-3'	7	22
5'-GTTGAGGGGCATCGTCGCGG-3'	8	23
5'-CGTTGAGGGGCATCGTCGCG-3'	9	24
5'-ACGTTGAGGGGCATCGTCGC-3'	10	25
5'-AACGTTGAGGGGCATCGTCG-3'	11	26
5'-TAACGTTGAGGGGCATCGTC-3'	12	27
5'-CTAACGTTGAGGGGCATCGT-3'	13	28
5'-GCTAACGTTGAGGGGCATCG-3'	14	29
5'-AGCTAACGTTGAGGGGCATC-3'	15	30
5'-AAGCTAACGTTGAGGGGCAT-3'	16	31
5'-GAAGCTAACGTTGAGGGGCA-3'	17	32

The protein-protein binding (carboxy terminal) domain of *myc* could also be altered, as follows. An oligomer with the antisense sequence 5'-TCC TCA TCT TCT TGT TCC TC-3' (SEQ ID NO: 33) targets base 6656, downstream of the splice acceptor at base 6654-5. Likely downstream cryptic SA sites are at bases 6704, 6710, and 6729 (AG at 6702-3; CAG at 6707-09; CAG at 6726-8; A branch point at 6684; pyrimidine-rich region starting at 6690). Of these, the first two are out of frame and the third is in frame. Use of the third site would be expected to produce a deletion of 75 base pairs from the mRNA, resulting in a 25 amino acid deletion in the protein-protein binding domain of the *myc* protein. This protein would be the converse dominant negative, as DNA binding may be possible but no *myc: max* transactivation is likely. The function of *myc* would be lost, but the *mad: max* heterodimers would be favored, so that the phenotype of differentiation and anti-apoptosis would be observed.

20 As noted above, oligomers targeted progressively farther downstream, relative to SEQ ID NO: 33, could also be used. This includes, for example, oligomers having a length of

about 18-20 contiguous nucleotides selected from the sequence 5'- AAC AAC ATC GAT
TTC TTC CTC ATC TTC TTG TTC CTC -3' (SEQ ID NO: 34; complementary to bases
6656-91 of the sequence given in Genbank Acc. No. J00120). In accordance with the
invention, an oligomer targeted far enough downstream to inhibit splicing at the first two
5 cryptic sites noted above could be effective to promote splicing at the third cryptic site.

D. Human p53 (GenBank X54156)

Like *c-myc*, p53 has a non-coding exon 1, a large intron 1 and an AUG start codon
near the SA site of exon 2. An oligomer targeted, for example, to the region having its 5'
10 end at base 11691, three bases downstream of the SA site (5'-CCC GGA AGG CAG TCT
GGC-3'; SEQ ID NO: 35) is expected to interfere with translation at the AUG initiation
site as well as the normal splicing of exon 2. As described for *c-myc* above, other suitable
oligomers include those targeted one or two bases downstream of the SA site, or targeted
progressively further downstream, e.g. starting at base 11691, 11692, etc., and targeting
15 some portion of the region between base 11689 (the first base of the normal exon 2) and
about base 11725. This includes, for example, oligomers having a length of about 18-20
contiguous nucleotides selected from the sequence 5'- TCC TCC ATG GCA GTG ACC
CGG AAG GCA GTC TGG CTG -3' (SEQ ID NO: 36; complementary to bases 11689-
11724 of the sequence given in Genbank Acc. No. X54156).

20 Cryptic SA sites are available at base 11761 (AG at 11759-60) and at base 11765
(CAG at 11762-4) (A branch point at 11736; pyrimidine run at 11750-57). The next
AUG, at base 11782, is out of frame, which will result in nonsense proteins.
Alternatively, initiation may begin in exon 3, producing truncated p53-type proteins
without the p53 amino terminus.

25 The following is an example in which targeting of the splice acceptor is especially
advantageous.

E. Human Abl (GenBank AJ131466)

30 Since the bcr gene breaks and fuses to abl, forming the bcr-abl fusion protein, in
chronic myeloid leukemia, it is a target of antisense inhibition. With respect to abl, fusion
can occur at various locations; i.e. there are breaks after exon 1, 2 and 3 of bcr that fuse to
Exon 2 of abl. However, by targeting the splice acceptor of abl, only one oligomer is
required for treatment of all CML patients.

35 The bcr-abl fusion point is at the junction of bases 373-374. Therefore, the abl splice

acceptor could be targeted by the following sequence, three bases downstream of this junction: 5'-CTA CTG GCC GCT GAA GGG C-3' (SEQ ID NO: 37).

Again, other oligomers targeting the region between the splice junction and about 35-40 bases downstream of the splice junction could also be used. This includes, for
5 example, oligomers having a length of about 18-20 contiguous nucleotides selected from the sequence 5'- GCT CAA AGT CAG ATG CTA CTG GCC GCT GAA GGG CTT -3' (SEQ ID NO: 38; complementary to bases 374-409 of the sequence given in Genbank Acc. No. AJ131466). Possible cryptic splice sites (both out of frame) include CAG motifs at 468-70, with an upstream pyrimidine region at 453-459 and an A branch point at 421,
10 and at 516-518, with an upstream pyrimidine region at 507-510 and an A branch point at 485.

F. HIV-1 (GenBank L39106)

This example illustrates a situation in which the method of the invention provides
15 particular advantages; that is, where both a target virus and the host (human) express a gene with the same mRNA sequences. The protein products are important to the function of both virus and host but serve different functions.

An mRNA sequence of HIV-1 rev, which encodes a protein critical for viral replication (see e.g. H Mitsuya *et al.*, *Science* **249**:1533-1543, 1990), was found to also
20 occur in the host, leading to toxic effects when this sequence was inhibited by antisense. Specifically, a phosphorothioate oligomer having the sequence 5'-TCG TCG GTC TCT CCG CTT CTT CTT GCC-3' (SEQ ID NO: 39) was used to inhibit HIV-1 rev (Matsukura *et al.*, *PNAS USA* **86**:4244-4248, 1989). This region of rev is highly conserved in HIV; accordingly, it was targeted so that a large variety of viral isolates might be inhibited.

25 However, the preclinical development of this 27-mer was ended when 2 of 3 Rhesus monkeys treated by continuous infusion died of what appeared to be opportunistic infections. An excessive endotoxin burden was suspected, so the experiment was repeated, with care taken to remove endotoxin. However, two cynomolgous monkeys also died on days 8 and 9 of the continuous infusion in the repeat study. As endotoxin was
30 effectively removed from consideration, immunosuppression was suggested as the cause. The white blood cell (WBC) count in the three Rhesus monkeys was 9.5 ± 0.7 prior to infusion with the antisense phosphorothioate to HIV-rev, and fell to 6.9 ± 0.6 during the infusion. There was no associated change in RBC or hematocrit. Further, surface marker studies for cells involved in immune response were influenced: CD2 was reduced from 88

to 76, CD8 fell from 45 to 36, and CD20 rose from 14 to 18.

A homologous region to HIV-*rev* observed in the genomes of humans and monkeys was reported in *J. Virology* 66:2170-2179 (1992), thus accounting for these toxic effects.

According to the present method, an alternative sequence could be selected which is less likely to interfere with host processes. In accordance with the present invention, HIV-*rev* could be suppressed as follows.

The protein is encoded by two exons: exon 1, 5493..5568, 76 bases; and exon 2, 7885..8180, 296 bases; 124 amino acids total (GenBank L39106). An antisense, RNase H- incompetent oligomer targeted to the region between the splice junction (i.e. the first base of normal exon 2) and about 35-40 bases downstream of the splice junction is expected to interfere with splicing of the pre-mRNA. As specified above, the 5' end of the targeted region is preferably 1 to about 25 bases downstream of the splice junction. An example of such an oligomer is a PMO targeted to base pairs 7885 - 7904, having the sequence 5'-CTC TGG TGG TGG GTA AGG GT-3' (SEQ ID NO: 40). Other candidates include oligomers having a length of about 18-20 contiguous nucleotides selected from the sequence 5'-CGG GTC TGT CGG GTT CCC TCT GGT GGT GGG TAA GGG T-3' (SEQ ID NO: 41; complementary to bases 7885-7921 of the sequence given in Genbank Acc. No. L39106).

The most likely site for a cryptic splice acceptor is at base 7975, where an AG sequence is preceded by multiple pyrimidines over 10 bases upstream from the AG. If this cryptic splice-acceptor is utilized, a deletion of 90 bases, or 30 amino acids, will result. This deletion will interfere with effective viral *rev* gene function.

VI. Analysis of Effects of Splice Acceptor Targeting

The effectiveness of a particular antisense sequence in producing splice variant mRNA may be determined by known analytical methods. For example, the presence or absence of the encoded, full-length protein and of truncated or other variant proteins can be monitored by standard techniques such as ELISA or Western blotting. Antibodies targeted to specific regions of the proteins, e.g. to the carboxy or amino terminus, can also be employed.

mRNA structure can be analyzed to evaluate antisense oligomer induced missplicing. The recovery of nuclear RNA is essential to observe intron-containing hnRNA, as the nucleus is the site of intron removal. Preparation of nuclear RNA is described in books such as "Molecular Cloning, A Laboratory Manual" (T. Maniatis, E.F. Fritsch and J.

Sambrook, eds., Cold Spring Harbor Press) or "Current Protocols in Molecular Biology" (F.M. Ausubel *et al.*, eds., John Wiley & Sons, Inc.). The analysis of hnRNA is best done by either Northern blot or S1 Mapping. The presence of particular splice variant mature mRNAs can be determined by carrying out PCR amplification using selected primer pairs

5 having sequences predicted to occur in a specific splice variant (or normal) processed mRNA. One primer is positioned in the SD (splice donor) exon upstream of the splice site, and the second is positioned in the SA (splice acceptor) exon, downstream of the splice site. Since the oligomer interferes with the SA site, the downstream primer should be more than 50 bases downstream from the SA splice site. Analysis of the PCR reactions

10 on agarose gels stained with ethidium bromide will reveal amplified bands which are smaller in size than bands observed from untreated control cells, as evidence of blocking of the SA site by the oligomer.

VII. Advantages of the Method

15 The present invention demonstrates that inhibition or alteration of protein expression can be achieved by antisense targeting of a region downstream of a splice acceptor, in the coding region of a pre-mRNA, using a non-RNase competent oligomer. The present compositions and methods have several advantages over prior art methods of antisense inhibition, in which the targeting antisense compound spans and hybridizes to a splice

20 acceptor sequence (e.g. Giles, cited above; Kole and Dominski, U.S. Patent No. 5,665,593).

One such advantage is that exon sequences tend to much more highly conserved among species than intron sequences. This allows for greater predictability in testing such methods on animal models.

25 In addition, greater flexibility is allowed in selecting a sequence for targeting, as the oligomer is not required to actually bind to a precise site such as a splice acceptor junction or an AUG start codon. The present invention thus increases the range of sequences which may be successfully targeted in an antisense application. Such flexibility can be advantageous in avoiding undesirable side reactions, such as caused by inadvertent

30 targeting of non-target proteins in a subject, or targeting of host proteins when attacking an infectious agent such as a virus or bacterium. This is demonstrated in Example F, above.

In the present strategy, in contrast to methods which target the splice junction directly, various regions of the exon downstream of the SA site may be blocked. This opens the possibility of producing different variant proteins, by directing splicing to different cryptic

splice sites downstream of the normal SA. As described above, certain variant proteins, such as dominant negative proteins, can have unique advantages. Accordingly, the antisense could be designed to promote splicing at a particular cryptic splice site, which would give rise to the desired variant protein, over others. For example, a less desirable cryptic splice site close to the normal SA site could be hindered in favor of a site further downstream.

Further benefits of flexibility of design may include convenience of synthesis or enhanced binding affinity. In addition, by using the present strategy of targeting fully within the exon, the target RNA can be identified by the antisense oligomer in either the nucleus or the cytoplasm, which can be advantageous for purposes of analysis.

VIII. Treatment Methods

In a related aspect, the invention includes a method of treating or preventing a disease state by inhibiting or altering expression of a target protein. Such disease states include viral, bacterial or fungal infections, cancerous tumors, and other conditions characterized by cellular proliferation, such as restenosis, hyperproliferative skin disorders, or inflammation. Proteins targeted, as noted above, include transcription factors, which include many oncogenes, receptor proteins, matrix proteins, and viral proteins. Inhibition of such proteins generally results in disruption of the cell cycle, viral replication, or other critical functions.

The method is carried out by administering to the subject an antisense oligomer 12 to 25 nucleotides in length and having (i) a base sequence complementary to a target region of a selected preprocessed mRNA coding for the target protein, where the 5' end of the target region is 1-25 bases downstream, and preferably 2-15 bases downstream, of a normal splice acceptor site in the preprocessed mRNA, and (ii) an uncharged morpholino backbone, preferably a phosphorodiamidate backbone as shown in Figure 2B-B, where X, Y, and Z are as defined above. Preferably, the compound also contains a moiety that enhances the solubility of the compound, preferably to a solubility in aqueous medium of between 25-50 mgs/ml or greater. An example is a polyethylene glycol (PEG) chain.

In general, the method comprises administering to a subject, in a suitable pharmaceutical carrier, an amount of the antisense agent effective to interfere with splicing at the normal splice acceptor site, and thus suppress normal expression of the protein. In a preferred embodiment, the method results in expression of a dominant negative variant of the protein. In one aspect of the method, the subject is a human subject.

Effective delivery of the antisense oligomer to the target mRNA is an important aspect of the method. PMOs have been shown to enter cells efficiently (see e.g. J Summerton *et al.*, *Antisense Nucleic Acid Drug Dev.* 7:63-70, 1997, and copending and co-owned U.S. provisional application 60/117,846). For use in antiviral treatment, various systemic routes of delivery, including oral and parenteral routes, e.g., intravenous, subcutaneous, intraperitoneal, and intramuscular, as well as inhalation, transdermal and topical delivery, can be used.

Typically, one or more doses of antisense oligomer are administered, generally at regular intervals for a period of about one to two weeks. Preferred doses for oral administration are from about 1 mg oligomer/patient to about 25 mg oligomer/patient (based on a weight of 70 kg). In some cases, doses of greater than 25 mg oligomer/patient may be necessary. For IV administration, the preferred doses are from about 0.5 mg oligomer/patient to about 10 mg oligomer/patient (based on an adult weight of 70 kg). Dosages will vary in accordance with such factors as the age, health, sex, size and weight of the patient, the route of administration, and the efficacy of the oligonucleotide agent with respect to the particular disease state. For treatment of infectious agents, a preferred dosage is typically that which is necessary to attain a concentration in the blood of from about 0.01 to about 1 μ M, and more preferably about 200-400 nM antisense oligomer. This concentration can be achieved in a variety of ways; doses of between about 0.05 and about 0.2mg/kg/hour by continuous IV infusion have been found to be acceptable. Greater or lesser amounts of oligonucleotide may be administered as required.

For treatment of hyperproliferative skin disorders, topical administration is indicated. In treatment of restenosis, delivery of the antisense oligomer to the affected cells, that is, to the site of arterial injury, is recommended. Delivery methods known in the field, such as those described in co-owned and copending U.S. application serial no. 09/493,427, can be used to deliver the oligomer to the site of angioplasty in a patient. Preferably, the oligomer is delivered concurrent with the angioplasty procedure. For an adult human, a recommended dosage is in the range of 1-25 μ mol of antisense oligomer, and preferably 2-15 μ mol. With respect to the surface area of tissue to be treated, an effective dose is typically in the range of 30 to 3000 μ g oligomer per cm^2 of vessel wall, and more preferably about 300 to 1500 μ g/ cm^2 . The patient may also be given the composition on a periodic basis after angioplasty, at a dosage level sufficient to further inhibit restenosis.

An effective *in vivo* treatment regimen using the antisense oligonucleotides of the

invention will vary according to the frequency and route of administration, as well as the condition of the subject under treatment. Optimum dosages for a given route can be determined by routine experimentation according to methods known in the art. Such *in vivo* therapy is generally monitored by tests appropriate to the particular type of ailment being treated, and a corresponding adjustment in the dose or treatment regimen can be made in order to achieve an optimal therapeutic outcome.

Entry of a morpholino oligomer into cells and binding to its target RNA sequence can be verified by techniques set forth in copending and co-owned U.S. provisional application 60/117,846, which is incorporated herein by reference. A morpholino antisense compound of the type disclosed herein, when administered *in vivo*, can be detected in the urine of the receiving subject in a heteroduplex form consisting of the antisense compound and its RNA complement. This verifies that the antisense compound has been taken up by the target tissue and allows the practitioner to monitor the effectiveness of the treatment method, e.g. the effectiveness of various modes of administration, and dosages giving maximal or near-maximal levels of heteroduplex in the urine.

IX. Formulations

A morpholino antisense oligonucleotide composition may be administered in any convenient physiologically acceptable vehicle. Examples of standard pharmaceutically accepted carriers include saline, phosphate buffered saline (PBS), water, aqueous ethanol, emulsions such as oil/water emulsions, triglyceride emulsions, wetting agents, tablets and capsules. It will be understood that the choice of suitable physiologically acceptable carrier will vary dependent upon the chosen mode of administration.

In general, in addition to the active compounds, the pharmaceutical compositions of the invention may contain suitable excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Suitable excipients include fillers such as sugars, for example, lactose, sucrose, mannitol or sorbitol, cellulose preparations, calcium phosphates, and binders such as starch, gelatin, methyl cellulose, hydroxypropyl methylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added, such as the above-mentioned starches as well as carboxymethyl starch, cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof. Auxiliaries include flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, and/or polyethylene

glycol.

For oral administration, dragee cores may be provided with suitable coatings which are resistant to gastric juices. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethyl-cellulose phthalate, are used. Concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinylpyrrolidone, polyethyleneglycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of the active compounds with a suppository base. Suitable suppository bases include natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols, or higher alkanols. In addition, it is possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable liquid formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble or water-dispersible form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, and/or dextran. The suspension may also contain stabilizers.

In addition to administration with conventional carriers, the active ingredients may be administered by a variety of specialized delivery techniques. For example, the compounds of the present invention may be administered encapsulated in liposomes. (See, *e.g.*,

Williams, S.A., *Leukemia* 10(12):1980-1989, 1996; Lappalainen *et al.*, *Antiviral Res.* 23:119, 1994; Uhlmann *et al.*, "Antisense Oligonucleotides: A New Therapeutic Principle", in *Chemical Reviews*, Volume 90, No. 4, pp 544-584, 1990; Gregoriadis, G., Chapter 14, "Liposomes", in *Drug Carriers in Biology and Medicine*, pp 287-341,

5 Academic Press, 1979.) The active ingredient, depending upon its solubility, may be present both in the aqueous phase and in the lipidic layer(s), or in what is generally termed a liposomal suspension. The lipidic layer generally comprises phospholipids, such as lecithin or sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other hydrophobic materials.

10 The diameters of the liposomes generally range from about 15 nm to about 5 microns.

Hydrogels may also be used as vehicles for antisense oligomer administration, for example, as described in WO 93/01286. Alternatively, the oligonucleotides may be administered in microspheres or microparticles. (See, *e.g.*, Wu GY and Wu CH, *J. Biol. Chem.* 262:4429-4432, 1987.) Such vehicles are particularly suited for topical

15 administration or in treating restenosis.

Transdermal delivery of antisense oligomers may be accomplished by use of a pharmaceutically acceptable carrier adapted for topical administration. One example of morpholino oligomer delivery is described in PCT patent application WO 97/40854.

Other sustained release compositions are also contemplated within the scope of this application. These may include semipermeable polymeric matrices in the form of shaped articles such as films or microcapsules.

20 Methods for preparing such dosage forms are known or will be apparent to those skilled in the art; for example, see *Remington's Pharmaceutical Sciences* (19th Ed., Williams & Wilkins, 1995). The pharmaceutical preparations are manufactured according to procedures well known in the art. For example, they may be made by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. The process to be used will depend ultimately on the physical properties of the active ingredient used.

30 MATERIALS AND METHODS

Oligomers

PMO's were synthesized at AVI BioPharma by methods described, for example, in Summerton and Weller, *Antisense & Nucleic Acid Drug Dev.* 7:187-95, 1993; U.S. Patent No. 5,185,444, 1997. The oligomers were purified by ion exchange chromatography and

analyzed for purity by high-performance liquid chromatography (HPLC) and mass spectrometry. The amount of full-length product was generally >90%. Before use, they were prepared as concentrated stock solutions with distilled water and stored at 4°C.

5 Cell Culture

Cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) or were derived in this laboratory. They were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F-12 supplemented with glutamine (2mM), streptomycin (100 µg/ml), and penicillin (100 U/ml). Dialyzed fetal bovine serum (FBS) was purchased from either Sigma (St. Louis, MO) or Hyclone (Ogden, UT). WI-38 and HeLa cells were cultured in 10% serum and NRK cells were cultured in 4% serum.

Recombinant plasmids and cell lines

15 The human *myc* genomic clone pHSR-1 was a deposit from M. Bishop to the ATCC. The 2.2-kilobase (kb) 5'-end was adapted for cloning into a luciferase vector by PCR using appropriate restriction sites incorporated into the primers (Scharf, 1990). The luciferase vector was adapted from one supplied by Clontech Inc. (Palo Alto, CA) to allow N-terminal fusion proteins with insect luciferase. Plasmids were introduced into HeLa
20 cells using the Lipofectin protocol and reagent from Life Science Technologies (Gaithersburg, MD) using the neomycin gene/Geneticin selection procedure (F Colbere-Garapin *et al.*, *J. Mol Biol.* **150**:1-14, 1981). The rabbit α -globin-luciferase construct-containing cell line has been described (M Partridge *et al.*, *Antisense & Nucleic Acid Drug Dev.* **6**:169-75, 1996).

25 [³H] Thymidine incorporation assay

Cells were trypsinized, counted, and plated at 400,000 cells/2 ml/well into 6-well dishes. On day 2, PMO antisense agents were added to the desired concentration, and the cells were scraped loaded (PL McNeil *et al.*, *J. Cell Biol.* **98**:1556-64, 1984; Partridge *et al.*,
30 cited above) with a rubber policeman using a gentle sweeping motion. The cell suspension was pipetted once to partly disaggregate any clumps formed, and 1 ml was transferred to a 24-well dish containing an additional 1 ml/well of fresh medium. For NRK cells, on day 4, 1 µCi of [³H]thymidine (DuPont, NEN, Wilmington, DE) (NET-027) was added per well, and 6 hours later, the cells were washed twice with phosphate-buffered

saline (PBS), precipitated with 5% trichloroacetic acid, washed a further two times with PBS, solubilized with 0.2 N NaOH/0.1% SDS, and the amount of radioactivity was incorporated into DNA quantitated in a scintillation counter. The quantitation procedure was identical for WI-38 cells except that the incubation period for incorporation was 15 hours. In general, each agent or concentration was assayed in duplicate, and the values were averaged. The duplicates were usually within 10% of each other.

Luciferase assay

The *myc*-luciferase HeLa cells were scrape loaded in a manner similar to the normal fibroblasts with the following changes. One million cells were plated into 6-well dishes, and on day 2 the entire 2 ml volume was transferred to another 6-well dish. Thirty hours later, the cells were collected and assayed for luciferase light production as described (Partridge *et al.*; 1996; Summerton *et al.*, 1997; cited above).

Cell cycle analysis

Cells were analyzed for cell cycle stage by flow cytometry. The cells were scrape loaded as described, and two wells were combined and replated in 10-cm dishes to obtain enough cells for analysis by fluorescence-activated cell sorting (FACS). Two days later, the cells were trypsinized, washed with PBS and resuspended in cold 80% ethanol for at least 2 hours. Following the fixation step, the cells were collected by centrifugation and stained with the DNA fluorochrome propidium iodide (Telford *et al.*, cited above). The ethanol-treated pellet was resuspended in 1 ml of 1 mM EDTA, 50 µg/ml propidium iodide, 1 µl/ml Triton X-100, and 10 µg/ml RNase A. After at least 1 hour at ambient temperature, the cell suspension was analyzed using a Coulter Epic XL-MCL flow cytometer (Coulter Electronics, Hialeah, FL) with an exciting wavelength of 488 nm. Data were analyzed using Phoenix Systems (San Jose, CA) multicycle program software.

mRNA reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

To examine the effects of Morpholino oligomers on *myc* RNA splicing, HeLa cells were plated in 6-well dishes at 1 million cells/well. The next day, oligomers at 20 µM final concentration were added to the medium and scrape loaded into the cells as detailed previously. Twenty-four hours later, the loaded cells were harvested by trypsinization and RNA prepared. Two wells were combined for each sample. Cytoplasmic (mature) RNA was extracted from the cell pellet by a Triton X-100 lysis procedure using a Qiagen Rneasy Mini Kit (Chartworth, CA) following the directions for "isolation of RNA from

the cytoplasm of animal cells." The RNA was eluted in 30 μ l of water with a yield of about 10-20 μ g.

- Six microliters of RNA (2-3 μ g) was reverse transcribed in a final 20 μ l reaction mixture with 1X PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) (Perkin-Elmer, Norwalk, CT) 1 mM of each deoxynucleotide triphosphate, 0.75 μ g 9-mer random primers, and 250 U Moloney murine leukemia virus (MmuLV) RT (New England Biolabs, Beverly, MA). After addition of enzyme, the reactions were incubated for 10 minutes at 25°C, following by 30 minutes at 42°C, and 4 minutes to denature the polymerase at 94°C.
- Exon 1-exon 2 PCR was performed using a two-step nested PCR procedure. Step 1 primers had the sequences 5'-CGG GCA CTT TGC ACT GAA ACT TAC AAC ACC (SEQ ID NO: 51) and 5'-GGT CGC AGA TGA AAC TCT GGT T (SEQ ID NO: 52). One microgram of each of the primers was added to the 20 μ l RT reaction, and the volume was adjusted to 100 μ l with 1X PCR buffer. Four units of Amplitaq (Perkin-Elmer) Taq polymerase were added, and 30 cycles were performed with steps of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 40 seconds. Step 2 used primer sequences of 5'-CTC CTT GCA GCT GCT TAG ACG CTG G (SEQ ID NO: 53) and 5'-GAA GGG TGT GAC CGC AAC GTA GGA G (SEQ ID NO: 54). The step 1 reaction mixture (4 μ l) was added to 96 μ l of 1X PCR buffer with 200 nM of each triphosphate and 1 μ g of each primer.
- Step 2 PCR conditions were 30 cycles of 94°C for 30 seconds, 68°C for 40 seconds, and 74°C for 30 seconds. Aliquots were then analyzed by agarose gel electrophoresis.

Sequence Table

SEQ ID NO	Sequence (5'→3')	Target [†]	Genbank Acc. No.	Location
1	CTGTGCTTAC/CGGGTTTTCACCTCCC	Rat <i>c-myc</i> (SD)	Y00396	2553-79
2	ATCGTCGTGACTGT/CTGTTGGAGGG	Rat <i>c-myc</i> (SA)		4140-64
3	GCTCACGTTGAGGGGCATCG	Rat <i>c-myc</i> (ds of SA)		4161-80
4	GGTCACTCAC/CGGTAGAGAA	Rat CYP3A2 (SD)	X62087	1155-74
5	GGGTTCCAAGT/CTATAAAGG	Rat CYP3A2 (SA)		1526-45
6*	TGTGTCTTTCCAG	Human androgen receptor exon 2	M35845	31-44
7*	TTTGGAGACTGCCAGGACCATG	"		45-67
8	CATGGTCCCTGGCAGTCTCC	"		48-67
9	TCAATGGGCAAAACATGGTCCCTGGCAGTCTCCAAA	"		45-80
10*	TTTGTGTCTCCAG	Human androgen receptor exon 3	M35846	28-43
11*	GGAAACAGAAGTACCTGTGCGCC	"		44-66
12	GGCGCACAGGTAATTCTG	"		49-66
13	AATCATTTCTGCTGGCGCACAGGTAATTCTGTTTCC	"		44-79
14	CCCCTGCAGCACGCGGGT	Human HCG- β	X00266	1321-38

		subunit		
15	GAGGCAGGGCCGGCAGGACCCCTGC AGCACGGGGT	"		1321-57
16	GGCATCGTCGCGGAGGCTG	Human <i>c-myc</i>	J00120	4506-25
17	GGGCATCGTCGCGGAGGCT	"		4507-26
18	GGGGCATCGTCGCGGAGGC	"		4508-27
19	AGGGGCATCGTCGCGGAGG	"		4509-28
20	GAGGGGCATCGTCGCGGAG	"		4510-29
21	TGAGGGGCATCGTCGCGGA	"		4511-30
22	TTGAGGGGCATCGTCGCGG	"		4512-31
23	GTTGAGGGGCATCGTCGCGG	"		4513-32
24	CGTTGAGGGGCATCGTCGCG	"		4514-33
25	ACGTTGAGGGGCATCGTCGC	"		4515-34
26	AACGTTGAGGGGCATCGTCG	"		4516-35
27	TAACGTTGAGGGGCATCGTC	"		4517-36
28	CTAACGTTGAGGGGCATCGT	"		4518-37
29	GCTAACGTTGAGGGGCATCG	"		4519-38
30	AGCTAACGTTGAGGGGCATC	"		4520-39
31	AAGCTAACGTTGAGGGGCAT	"		4521-40
32	GAAGCTAACGTTGAGGGGCA	"		4522-41
33	TCCTCATCTCTTGTTCCTC	"		6656-75
34	AACAACATCGATTCTCTCATCTTC TTGTTCCTC	"		6656-91
35	CCCGGAAGGCACTCTGGC	Human p53	X54156	11691-708
36	TCCTCCATGGCAGTGACCCGGAAGGC AGCTCGCTG	"		11689-724
37	CTACTGGCCGCTGAAGGGC	Human abl (ds of bcr-abl fusion point)	AJ131466	376-94
38	GCTCAAAGTCAGATGCTACTGGCCGC TGAAAGGGCTT	"		374-409
39	TCGTGGTCTCTCCGTCTCTCTTGCC	HIV-1 rev (prior art)	U69590	5517-43
40	CTCTGGTGGTGGGTAAGGGT	HIV-1 rev	L39106	7885-7904
41	CGGGTCTGTGGGTTCCCTCTGGTGGT GGGTAAGGGT	"		7885-7921
42	GGGGCAUCGUCGUGACUGU/CUGUUG GAGGG	Rat <i>c-myc</i> (SA)	Y00396	4140-69
43	CGUCGUGACUGU/CUGUUGGAGG	"	Y00396	4141-62
44	CGTCGTGACTGT/CTGTTGGAGG	"	Y00396	4141-62
45	GGCAUCGUCGCGGAGGCGUG/CUGGA GCG	Human <i>c-myc</i> (SA)	J00120	4498-4505
46	CCGCGACAUAGGACGGAGAGCAGAG CCC	Rat <i>c-myc</i>	Y00396	4364-91
47	ACTGTGAGGGCGATCGCTGC (scrambled)	derived from SEQ ID NO: 25		
48	ACGATGAGTGGCATAGTCGC (3 mismatches)	derived from SEQ ID NO: 25		
49	CTCCGCAATGCTGAAAGGTG	Rat BCL-2 (cntrl)		
50	GGCGUGCCUCAAACAUGGUGGCGG	Rat PCNA-1 (cntrl)		
51	CGGGCACTTTGCACTGAAACTTACAA CACC	primer sequence		
52	GGTCGCAGATGAAACTCTGGTT	"		
53	CTCCTTGACGCTGCTTAGACGCTGG	"		
54	GAAGGGTGTGACCGCAACGTAGGAG	"		

* native sequence, not antisense

† unless otherwise indicated, antisense target is downstream (ds) of splice acceptor (SA) junction; SD = splice donor junction

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An antisense compound composed of morpholino subunits joined by phosphorous containing intersubunit linkages and a base sequence between 12 and 25 nucleotide bases in length which is complementary to a target region of a preprocessed mRNA having multiple exons that encode multiple domains in a protein selected from the group consisting of human abl, human p53, human androgen receptor protein, human chorionic gonadotropin (hCG), and HIV-1 rev,
 wherein the 5' end of the target region for the antisense compound is 1-25 bases downstream of a normal splice acceptor site within a selected exon in the preprocessed mRNA and the compound alters splicing of the selected protein's preprocessed mRNA to form a variant mRNA encoding a variant protein with altered activity.
2. The compound of claim 1, wherein the 5' end of the target region for the antisense compound is 2-20 bases downstream of a normal splice acceptor site.
3. The compound of claim 2, wherein the 5' end of the target region is 10-15 bases downstream of the normal splice acceptor site.
4. The compound of any one of claims 1 to 3, wherein the variant protein is truncated in a protein domain encoded by at least one exon.
5. The compound of any one of claims 1 to 4, wherein the morpholino subunits are linked together by uncharged phosphorous-containing linkages, one to three atoms long, joining the morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and further comprises, attached to each morpholino subunit, a purine or pyrimidine base-pairing moiety effective to bind, by base specific hydrogen bonding to a base in a polynucleotide.
6. The compound of any one of claims 1 to 5, which targets the splice acceptor in exon 2 of the preprocessed mRNA for human androgen receptor.
7. The compound of any one of claims 1 to 5, which targets the splice acceptor in exon 3 of the preprocessed mRNA for hCG.

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8. The compound of any one of claims 1 to 5, which targets the splice acceptor in exon 2 of the preprocessed mRNA for human p53.
9. The compound of any one of claims 1 to 5, which targets the splice acceptor in exon 2 of the preprocessed mRNA for human abl.
10. The compound of any one of claims 1 to 5, which targets the splice acceptor in exon 2 of the preprocessed mRNA for the HIV-1 rev protein.
- 10 11. The compound of any one of claims 1 to 5, wherein the base sequence of the antisense compound is selected from the group consisting of:
- (a) for hCG, β subunit: a contiguous 18- to 20-nucleotide sequence contained within SEQ ID NO:15;
- (b) for human androgen receptor: a contiguous 18- to 20-nucleotide
- 15 sequence contained within a sequence selected from SEQ ID NO:9 or SEQ ID NO:13;
- (c) for human p53: a contiguous 18- to 20-nucleotide sequence contained within SEQ ID NO:36;
- (d) for human abl: a contiguous 18- to 20-nucleotide sequence contained
- 20 within SEQ ID NO:38; and
- (e) for HIV-1 rev: a contiguous 18- to 20-nucleotide sequence contained within SEQ ID NO:41.
12. Use of an antisense compound composed of morpholino subunits joined by phosphorous-containing backbone linkages and a base sequence between 12 and 25 nucleotide bases in length which is complementary to a target region of a selected preprocessed mRNA having multiple exons that encode multiple domains in a selected protein, wherein the 5' end of the target region for the antisense compound is 1-25 bases downstream of a normal splice acceptor site within a selected exon in the
- 30 preprocessed mRNA and the compound alters splicing of the protein's preprocessed mRNA to generate a variant mRNA encoding a variant form of the protein with altered activity,
- for preparation of a medicament for use in producing, in eukaryotic cells, the variant protein with altered activity for treating a disease affected by the selected
- 35 protein.

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13. The use of claim 12, wherein the 5' end of the target region for the antisense compound is 2-20 bases downstream of a normal splice acceptor site.
14. The use of claim 12, wherein the 5' end of the target region is 10-15 bases downstream of the normal splice acceptor site.
15. The use of any one of claims 12 to 14, wherein the variant protein is truncated in a protein domain encoded by at least one exon.
16. The use of any one of claims 12 to 15, wherein said morpholino subunits are linked together by uncharged phosphorous-containing linkages, one to three atoms long, joining the morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and further comprises, attached to each morpholino subunit, a purine or pyrimidine base-pairing moiety effective to bind, by base specific hydrogen bonding to a base in a polynucleotide.
17. The use of any one of claims 12 to 16, wherein the protein is selected from the group consisting of human abl, human p53, human androgen receptor, hCG, and HIV-1 rev.
18. The use of any one of claims 12 to 16, wherein a splice acceptor site downstream of the normal splice acceptor site of the selected exon is whole multiple of three bases downstream of the normal splice acceptor site.
19. The use of any one of claims 12 to 16, wherein the selected protein has multiple distinct DNA binding or protein binding regions, and the variant mRNA encodes a variant protein in which the binding region is disabled.
20. The use of claim 19, wherein the variant protein is a dominant negative protein.
21. The use of any one of claims 12 to 20, wherein the selected protein and corresponding antisense base sequence are selected from the group consisting of:
- (a) human chorionic gonadotropin, β subunit: a contiguous 18-to 20-nucleotide sequence contained within SEQ ID NO: 15;
 - (b) human androgen receptor: a contiguous 18-to 20-nucleotide sequence selected contained within SEQ ID NO: 9 or SEQ ID NO: 13;

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(c) human p53 : a contiguous 18-to 20-nucleotide sequence contained within
SEQ ID NO: 36;

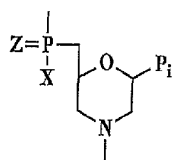
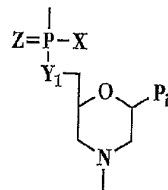
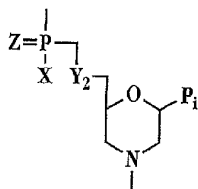
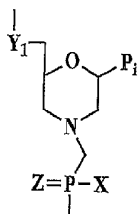
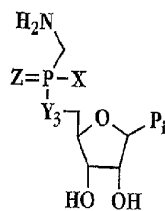
(d) human abl : a contiguous 18-to 20-nucleotide sequence contained within
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5 (e) HIV-1 rev: a contiguous 18-to 20-nucleotide sequence contained within
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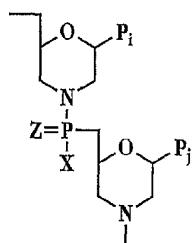
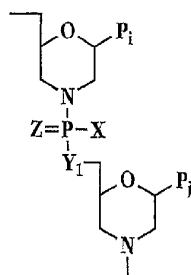
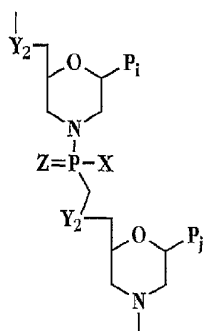
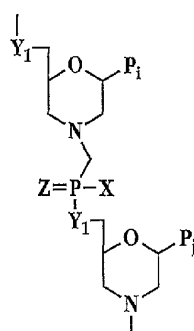
22. A method of producing in a eukaryotic cell a variant protein with altered activity
for treating a disease affected by the protein, the method comprising administering to
10 the subject an antisense compound composed of morpholino subunits joined by
phosphorous-containing backbone linkages and a base sequence between 12 and 25
nucleotide bases in length which is complementary to a target region of a selected pre-
processed mRNA having multiple exons and that encode multiple domains in a selected
protein, wherein the 5' end of the target region for the antisense compound is 1-25
15 bases downstream of a normal splice acceptor site within a selected exon in the
preprocessed mRNA and the compound alters splicing of the proteins preprocessed
mRNA to generate a variant mRNA encoding a variant form of the protein with altered
activity.

20 23. An antisense compound composed of morpholino subunits joined by
phosphorous containing intersubunit linkages and a base sequence between 12 and 25
nucleotide bases in length which is complementary to a target region of a preprocessed
mRNA having multiple exons that encode multiple domains in a protein selected
from the group consisting of human abl, human p53, human androgen receptor
25 protein, human chorionic gonadotropin (hCG), and HIV-1 rev, wherein the 5' end of
the target region for the antisense compound is 1-25 bases downstream of a normal
splice acceptor site within a selected exon in the preprocessed mRNA and the
compound alters splicing of the selected protein's preprocessed mRNA to form a
variant mRNA encoding a variant protein with altered activity, substantially as
30 hereinbefore described.

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**Fig. 1A****Fig. 1B****Fig. 1C****Fig. 1D****Fig. 1E**

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**Fig. 2A-A****Fig. 2B-B****Fig. 2C-C****Fig. 2D-D/E-E**

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