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(54) **SPLICE-REGION ANTISENSE COMPOSITION AND METHOD**

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(57) **ABSTRACT**

Antisense compositions targeted against an mRNA sequence coding 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.

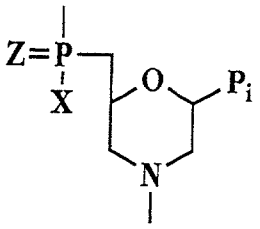

Fig. 1A

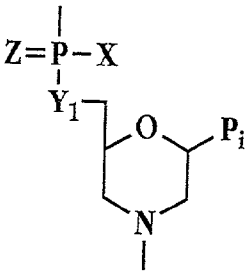

Fig. 1B

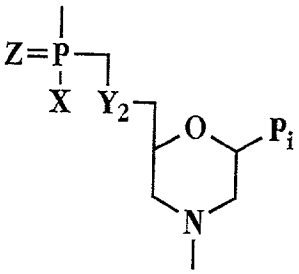

Fig. 1C

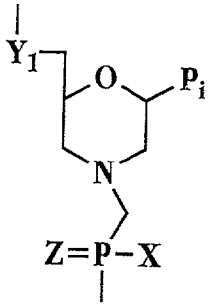

Fig. 1D

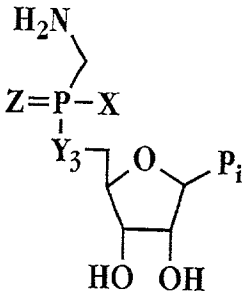

Fig. 1E

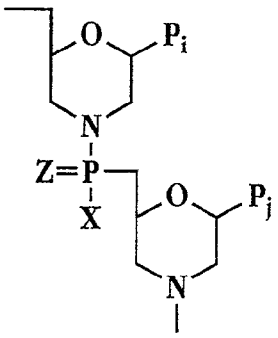

Fig. 2A-A

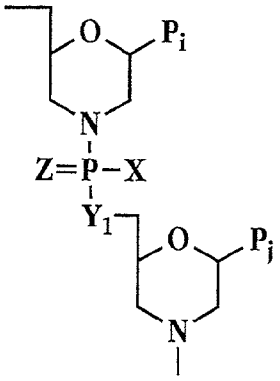

Fig. 2B-B

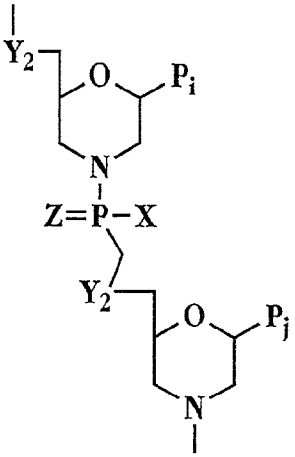

Fig. 2C-C

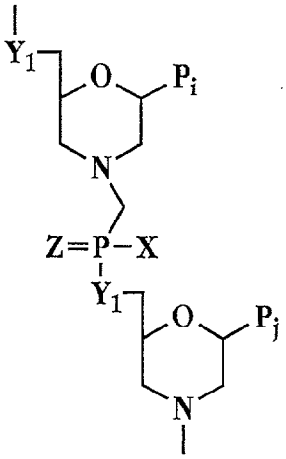

Fig. 2D-D/E-E

SPLICE-REGION ANTISENSE COMPOSITION AND METHOD

[0001] This application claims priority to U.S. provisional application serial no. 60/202,376, filed May 4, 2000, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] 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

[0003] 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.

[0004] 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. Pat. 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. R V 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.

SUMMARY OF THE INVENTION

[0005] 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:

[0006] (a1): an uncharged morpholino backbone;

[0007] (a2): a base-sequence length of between 12 and 25 nucleotide bases; and

[0008] (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, and having the properties that:

[0009] (b1): the compound is taken up by eukaryotic cells;

[0010] (b2): the compound hybridizes to the target region of preprocessed mRNA in such cells, and

[0011] (b3): the compound so hybridized to the target pre-mRNA prevents splicing at the 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.

[0012] 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.

[0013] In one embodiment, the compound has intersubunit linkages selected from the group consisting of the structures presented in FIGS. **2**AA-**2**EE. In preferred embodiments, the linkages are selected from a phosphorodiamidate linkage as represented at **FIG. 2**B-B, where X=NH_2, NHR, or NRR', Y=O, and Z=O, and an alternate phosphorodiamidate linkage as represented at **FIG. 2**B-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, 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.

[0014] 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.

[0015] 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.

[0016] 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-βsubunit, androgen receptor protein, and HIV-1 rev.

[0017] 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.

[0018] 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:

[0019] (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;

[0020] (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;

[0021] (c) human p53: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 36; e.g. SEQ ID NO: 35;

[0022] (d) human abl: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 38; e.g. SEQ ID NO: 37; and

[0023] (e) HIV-1 rev: a contiguous 18- to 20-nucleotide sequence selected from SEQ ID NO: 41; e.g. SEQ ID NO: 40.

[0024] These and other objects and 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **FIG. 1** shows several preferred subunits having 5-atom (A), six-atom (B) and seven-atom (C-E) linking groups suitable for forming polymers; and

[0026] FIGS. **2**A-A to **2**E-E show the repeating subunit segment of exemplary morpholino oligonucleotides, designated A-A through E-E, constructed using subunits A-E, respectively, of **FIG. 1**.

DETAILED DESCRIPTION OF THE INVENTION

[0027] I. Definitions

[0028] The terms below, as used herein, have the following meanings, unless indicated otherwise:

[0029] "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.

[0030] As used herein, the terms "compound", "agent", "oligomer" and "oligonucleotide" may be used interchangeably with respect to the antisense oligonucleotides of the invention.

[0031] As used herein, a "morpholino oligomer" refers to a polymeric molecule having a backbone which supports bases capable of hydrogen bonding to typical 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 FIGS. **1**A-E, with the linkages shown in FIGS. **2**A-A to **2**E-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).

[0032] Subunit A in **FIG. 1** contains a 1-atom phosphorous-containing linkage which forms the five atom repeating-unit backbone shown at A-A in **FIG. 2**, where the morpholino rings are linked by a 1-atom phosphoamide linkage.

[0033] A preferred morpholino oligonucleotide is composed of morpholino subunit structures of the form shown in **FIG. 2**B-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 Pi and Pj 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 **FIG. 2**B-B, where X=NH_2, NHR, or NRR', Y=O, and Z=O, and an alternate phosphorodiamidate linkage as represented at **FIG. 2**B-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. (An example of such a combination would be—$(CH_2)_3(CH_2CH_2O)_3$–). 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=$N(CH_3)_2$, 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, and pyridine.

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