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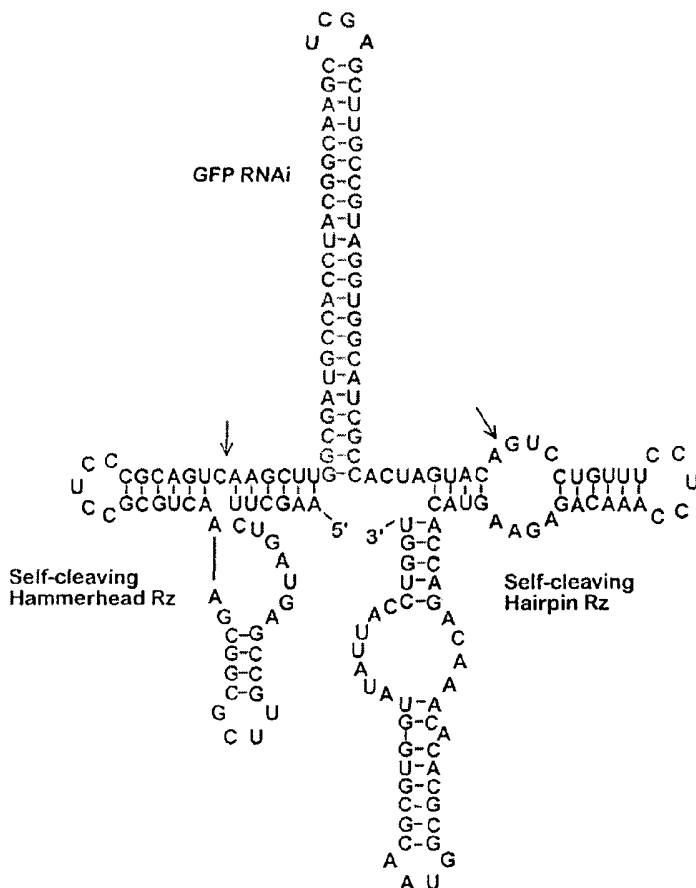
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(54) Title: INHIBITION OF GENE EXPRESSION AND THERAPEUTIC USES THEREOF



(57) Abstract: RNA interference (RNAi) has become a powerful tool for blocking gene expression in mammals and mammalian cells. This approach requires the delivery of small interfering RNA (siRNA) either as RNA itself or as DNA, using an expression plasmid or virus and the coding sequence for small hairpin RNAs that are processed to siRNAs. Current expression systems for the production of siRNA *in vivo* rely on RNA polymerase III promoters. These are difficult to regulate and leave most of the RNA in the nucleus of the cell where it is inactive for RNA interference. The invention provides a DNA cassette for the cloning of small hairpin sequences which permit their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and permits the use of regulated and tissue specific promoters for gene expression.



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INHIBITION OF GENE EXPRESSION AND THERAPEUTIC USES THEREOF

FIELD OF THE INVENTION

[0001] The invention provides compositions and methods for highly selective targeting of heterologous nucleic acid sequences. The oligonucleotides are siRNA's which bind in a sequence dependent manner to their target genes and inhibit expression of undesired nucleic acid sequences in a target cell. When administered into cells, siRNA's cause elimination or degradation of a non-essential extra-chromosomal genetic element. Moreover, siRNA's can specifically and selectively kill human cells if the target is present in their genomes and its product is required for viability.

BACKGROUND

[0002] In 1994, Nilsson and colleagues described an *in situ* hybridization technique, designated "padlock probes", which can detect single base mutations yet be seen at the light microscope level (Nilsson, M. *et al.* "Padlock probes: circularizing oligonucleotides for localized DNA detection". *Science* 265, 2085-8 (1994). Padlock probes are large oligonucleotides, whose arms are complementary to, and wrap around the target DNA in an end-to-end orientation, and are then ligated if a perfect match exists between the arms and target. Since both arms are typically about twenty bases each, together they are expected to wrap around a DNA target approximately four times before being locked through ligation (one turn per ~10 bases). In this way they are inextricably bound to the target (hence "padlock"), permitting highly stringent washing prior to detection, using either the biotin molecules in the non-complementary backbone or through rolling circle amplification.

[0003] With many diseases, patients exist as cell chimeras, in that they have acquired a second cell population (e.g. malignant cells, bacterial cells, HIV infected cells). In each case, this second cell population contains an additional gene or genes, which not only define these cells as unique, but also could be used to target this second cell population in the treatment of a patient.

[0004] While existing approaches to target cells based on their genotype is limited, some molecular based approaches have been developed. These include antisense RNA [(Izant, J.G. & Weintraub, H. *Science* 229, 345-52. (1985); Detrick, B. *et al.* *Invest. Ophthalmol. Vis. Sci.* 42, 163-9. (2001); Miller, P.S., Cassidy, R.A., Hamma, T. & Kondo, N.S. *Pharmacol. Ther.* 85, 159-63. (2000)], triplex DNA [(Blume, S.W., Gee, J.E., Shrestha,

K. & Miller, D.M. *Nucleic Acids Res* 20, 1777-84. (1992); Chan, P.P. & Glazer, P.M. *J. Mol. Med.* 75, 267-82. (1997); Cassidy, R.A., Kondo, N.S. & Miller, P.S. *Biochemistry* 39, 8683-91. (2000)], ribozymes [(Beaudry, A.A. & Joyce, G.F. *Science* 257, 635-41. (1992); Joyce, G.F. *Science* 289, 401-2. (2000)], "suicide" gene therapy [(Shimura, H. *et al.* *Cancer Res.* 61, 3640-6. (2001); Black, M.E., Kokoris, M.S. & Sabo, P. *Cancer Res.* 61, 3022-6. (2001)], and inhibitory RNA [(Elbashir, S.M. *et al.* *Nature* 411, 494-8 (2001); Brummelkamp, T.R., Bernards, R. & Agami, R. *Science* 296, 550-3 (2002)].

[0005] There is a need in the art to selectively target foreign genetic material, whether integrated or present as an extra-chromosomal episome, to be tissue and cell specific and to inhibit nucleic acid synthesis and resulting in selective cell death.

SUMMARY

[0006] Sequence specific siRNA bind to a target nucleic acid molecule, inhibiting the expression thereof. siRNA's are effective in the treatment of abnormal cell growth and diseases caused by infectious disease agents. Compositions for delivery of siRNA and methods thereof are provided.

[0007] In a preferred embodiment, tissue specific promoters are used for the expression of nucleic acids encoding siRNA's of the invention.

[0008] In another preferred embodiment, expression and intracellular processing of the small hairpin sequences is accomplished by RNA polymerase II.

[0009] In another preferred embodiment, vectors expressing siRNA's include, but are not limited to cloning vectors and virus delivery systems including plasmids, viral vectors such as: herpes virus, lentivirus, adenovirus, and adeno associated virus (AAV). Preferably, the vector is an AAV vector. In a preferred embodiment, the nucleic acid is operably linked to a regulatory region comprising a regulatable promoter, an inducible promoter, a tissue specific promoter, for example, a neural cell-specific promoter or a viral promoter.

[00010] In yet another preferred embodiment, a composition comprises a vector expressing an siRNA precursor, wherein said desired RNA precursor is present between a 3' region and a 5' region, wherein said 3' region and said 5' region form an intramolecular stem with each other comprising at least about 15 base pairs. Preferably, the RNA comprises hammerhead and hairpin ribozymes flanking a cloning site of a hairpin structure and the desired RNA sequences are complementary to a target RNA sequence.

[00011] In another preferred embodiment, a vector encoding a transcribed isolated RNA molecule, comprises a desired RNA portion, wherein said desired RNA portion is present

between a 3' region and a 5' region, wherein said 3' region and said 5' region form an intramolecular stem with each other comprising at least about 15 base pairs, and wherein the RNA molecule is transcribed by an RNA polymerase. Preferably, the RNA polymerase is RNA polymerase II and the vector is an adenovirus-associated vector.

[00012] In one aspect of the invention, the vector comprises regulated and/or tissue specific promoters operably linked to the desired RNA and the vector further comprises signal sequences for intracellular trafficking of the siRNA.

[00013] In another preferred embodiment, a composition comprises an siRNA that targets heterologous oligonucleotide sequences wherein binding of targeted oligonucleotides by the siRNA inhibits expression of a target gene.

[00014] In another embodiment, a cell in culture comprises a vector encoding a transcribed RNA molecule comprising a desired RNA portion, wherein said desired RNA portion is present between a 3' region and a 5' region, wherein said 3' region and said 5' region form an intramolecular stem with each other comprising at least about 15 base pairs and the RNA molecule is transcribed by RNA pol II.

[00015] In another preferred embodiment, siRNA precursors are coupled to RNA transport signals such as introns, and viral sequence elements, such as WPRE and CTE that permit the transport of RNA out of the nucleus and into the cytoplasm where RNA interference occurs.

[00016] In particular, the invention provides methods of inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, comprising: targeting the nucleic acid molecule with an oligonucleotide; and, binding of the oligonucleotide to the target nucleic acid molecule.

[00017] In another aspect of the invention, the siRNA oligonucleotide hybridizes with genomic target molecules as well as episomal structures. In another preferred embodiment, the target nucleic acid molecule in a cell is expressed in a disease state or is a foreign nucleic acid molecule. The disease state is cancer and/or an infectious disease organism, such as a virus. Other infectious disease organisms include protozoa or fungi.

[00018] In another preferred embodiment, the siRNA oligonucleotide comprises a total of from about 30 to about 60 base units, more preferably, a total of from about 60 to about 120 base units, most preferably, the siRNA oligonucleotide comprises a total of from about 42 to about 52 base units. However, different sizes of an siRNA can be designed depending on the target nucleic acid sequence.

[00019] Preferably the siRNA oligonucleotide has equal or higher specificity and affinity for a target oligonucleotide sequence than the complementary target oligonucleotide sequence. In one aspect, the association constant (K_a) of the oligonucleotide for the target nucleic acid molecule is higher than the association constant of the complementary strands of a double stranded molecule. In another aspect, the association constant (K_a) of the oligonucleotide for the target nucleic acid molecule is higher than a disassociation constant (K_d) of the complementary strand of the target sequence in a double stranded molecule.

[00020] In another embodiment, the siRNA oligonucleotide can bind to a wild type gene sequence and any alleles or variants thereof. This is useful in knock out experiments wherein the knock-down of wild type genes allows one of skill in the art to determine functions of these genes and also the functional domains of each. Preferably, the siRNA oligonucleotide binds to double-stranded RNA target molecules as well as single-stranded RNA targets, messenger RNA and/or RNA secondary structures. The invention may be used against protein coding gene products as well as non-protein coding gene products. Examples of non-protein coding gene products include gene products that encode ribosomal RNAs, transfer RNAs, small nuclear RNAs, small cytoplasmic RNAs, telomerase RNA, RNA molecules involved in DNA replication, chromosomal rearrangement of, for instance immunoglobulin gene products, etc. It may also include introns, or regions between gene products.

[00021] In a preferred embodiment, the invention provides a method for selectively treating cells comprising an infectious disease organism, comprising: administering to the cells an oligonucleotide sequence that is complementary to a target sequence of an infectious disease organism, or the cells comprising an oligonucleotide sequence of an infectious disease organism.

[00022] Preferably, the cells are mammalian cells and the cells are infected with a virus, protozoa or fungi. The cells do not have to be actively replicating and can be in any one of G1, S, M, or G2 stage of a cell cycle.

[00023] In another preferred embodiment of the invention the siRNA oligonucleotide binds to a wild type infectious disease organisms' target gene sequence and any alleles or variants thereof. The foreign target nucleic acid molecule can be single-stranded RNA targets, double-stranded RNA target molecules as well as single or double stranded viral RNA targets, messenger RNA and/or RNA secondary structures. The siRNA oligonucleotide preferably hybridizes with genomic target molecules as well as episomal structures.

[00024] In another preferred embodiment, the invention provides a method for treating a mammal suffering from or susceptible to an infectious disease or cancer, the method

comprising: administering to the mammal a therapeutically effective amount of an oligonucleotide. Preferably, the administered oligonucleotide hybridizes to the gene to inhibit expression thereof and/or results in inhibition of gene expression. Preferably, the infectious disease is caused by or associated with a virus, protozoa or fungi. In one aspect, the infectious agent is present in any tissue or organ of a mammal and the infectious agent is associated with undesired expression of at least a portion of a sequence, examples of which are shown in the tables and/or variants thereof.

[00025] In other preferred embodiment, siRNAs bind to repressor gene products and inhibit the activity of repressor molecules. For example, in the treatment of myocardial disease an inotropic effect is desired. Pharmacological therapies have been directed toward increasing the force of contraction of the heart (by using inotropic agents such as digitalis and β -adrenergic receptor agonists), reducing fluid accumulation in the lungs and elsewhere (by using diuretics), and reducing the work of the heart (by using agents that decrease systemic vascular resistance such as angiotensin converting enzyme inhibitors). siRNAs that inhibit the production of molecules which repress the activity of gene products in a disease state are well-within the scope of the invention.

[00026] In another preferred embodiment, siRNAs are used in the treatment of individuals who are hypersensitive or allergic to certain allergens. siRNAs are produced which prevent the expression of IgE molecules specific for certain allergens. Such siRNAs inhibit the rearrangement of immunoglobulin gene products with a specificity for such allergens.

[00027] The invention may be used against protein coding gene products as well as non-protein coding gene products. Examples of non-protein coding gene products include gene products that encode ribosomal RNAs, transfer RNAs, small nuclear RNAs, small cytoplasmic RNAs, telomerase RNA, RNA molecules involved in DNA replication, chromosomal rearrangement of, for instance immunoglobulin gene products, etc.

[00028] In another preferred embodiment, the siRNAs inhibit the expression of a target nucleic molecule in cells of in an organism in need of treatment. The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. For example, a plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

[00029] Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

[00030] Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphelenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynychus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

[00031] The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

[00032] In accordance with the invention, the siRNA is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target gene products are listed for illustrative purposes: developmental gene products (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation

factors and their receptors, neurotransmitters and their receptors); oncogene products (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor gene products (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

[00033] Other aspects of the invention are discussed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

[00034] The invention is pointed out with particularity in the appended claims. The above and further advantages of this invention may be better understood by referring to the following description taken in conjunction with the accompanying drawings, in which:

[00035] Figure 1 is a schematic illustration of an siRNA-ribozyme cassette of the invention.

[00036] Figure 2 is a schematic illustration of a vector encoding an siRNA-ribozyme cassette of the invention.

[00037] Figure 3 is a graph showing the effect of functional siRNA ribozyme cassette in cultured cells transfected with the plasmid vector shown in Fig. 2.

[00038] Figure 4 shows the results obtained when an AAV virus expressing the siRNA-ribozyme cassette directed to tyrosine hydroxylase mRNA is delivered to the brain of a rat.

[00039] Figures 5A to 5G is an image showing the results obtained from a vector expressing the ribozyme-siRNA cassette when transduced into rat brain substantia nigra. Figure 5A is a low magnification image of transduced rat brain substantia nigra. Figure 5B shows an image of transduced cells fluorescing green due to the GFP gene present in the AAV vector that also expresses an shRNA cassette specific for tyrosine hydroxylase expressed from a pol II promoter (CBA). Figure 5C shows that the red fluorescence is from immunoreactive tyrosine hydroxylase (TH), a bio-marker for all dopaminergic neurons in the nigro-striatal tract. Figures 5D to 5F are higher magnification images.

DETAILED DESCRIPTION

[00040] The invention provides compositions and methods for delivery of molecules that selectively kill cells based on their genotype, tumorigenicity, and/or heterologous sequences. The invention provides particular method for delivery of small hairpin precursors of siRNA so that they may be expressed using pol II promoters, especially regulatable pol II promoters, such as those controlled by tetracycline, rapamycin ecdysone etc. In particular, siRNA's bind specifically to their gene targets, thereby inhibiting expression of undesirable molecules. When transformed into a mixed population of cells, where only one cell type possesses the target, siRNA's selectively kill only the target bearing cell population. siRNA's will kill cells irrespective of their transcriptional status and depending on whether an essential protein has been inhibited. The siRNA's are active in eukaryotic cells.

Definitions

[00041] Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

[00042] As used herein, the term "ribozymes" refers to linear oligonucleotides with a loop structure, are catalytic nucleic acids, and are designed to inactivate specific mRNA.

[00043] "Synthetic catalysts" are catalysts designed according to the principles set forth herein based on the "hairpin" model to bind to and cleave a selected target sequence in a selected RNA substrate. "Synthetic catalysts" include catalysts synthesized *in vitro* and catalysts synthesized *in vivo*. In particular, "synthetic catalysts" include catalysts produced by hosts transformed by a vector comprising a sequence coding for the catalyst. An example of the ribozyme-siRNA is described in the examples which follow.

[00044] As used herein, the term "DNA repair gene" refers to a gene that is part of a DNA repair pathway, that when altered, permits mutations to occur in the DNA of the organism.

[00045] As used herein, the terms "exon" and "intron" are art-understood terms referring to various portions of genomic gene sequences. "Exons" are those portions of a genomic gene sequence that encode protein. "Introns" are sequences of nucleotides found between exons in genomic gene sequences. The siRNA's can be targeted to exons and /or to introns.

[00046] As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

[00047] The term "promoter," "promoter element," or "promoter sequence" as used herein, refers to a nucleic acid sequence which when ligated to a nucleotide sequence of interest is capable of controlling transcription of the nucleotide sequence of interest. A promoter is typically, though not necessarily, located 5' (i.e., upstream) of a nucleotide sequence of interest whose transcription it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. In a preferred embodiment, the RNA polymerase is RNA Pol II.

[00048] Promoters may be inducible, constitutive or regulatable. The term "constitutive" when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a stimulus (e.g., heat shock, chemicals, etc.). In contrast, a "regulatable" promoter is one which is capable of directing a level of transcription of an operably linked nucleic acid sequence in

the presence of a stimulus (e.g., heat shock, chemicals, etc.) which is different from the level of transcription of the operably linked nucleic acid sequence in the absence of the stimulus.

[00049] As used herein, the term "adeno-associated virus (AAV) vector" refers to a vector derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAVX7, etc. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, e.g. the rep and/or cap genes, but retain functional flanking ITR sequences. AAV vectors can be constructed using recombinant techniques that are known in the art to include one or more heterologous nucleotide sequences flanked on both ends (5' and 3') with functional AAV ITRs. In the practice of the invention, an AAV vector can include at least one AAV ITR and a suitable promoter sequence positioned upstream of the heterologous nucleotide sequence and at least one AAV ITR positioned downstream of the heterologous sequence. A "recombinant AAV vector plasmid" refers to one type of recombinant AAV vector wherein the vector comprises a plasmid. As with AAV vectors in general, 5' and 3' ITRs flank the selected heterologous nucleotide sequence. An exemplary AAV vector is described in the Examples which follow.

[00050] As used herein, the term "infectious agent" refers to an organism wherein growth/multiplication leads to pathogenic events in humans or animals. Examples of such agents are: bacteria, fungi, protozoa and viruses.

[00051] As used herein, the term "oligonucleotide specific for" refers to an oligonucleotide having a sequence (i) capable of forming a stable complex with a portion of the targeted gene, or (ii) capable of forming a stable duplex with a portion of a mRNA transcript of the targeted gene.

[00052] As used herein, the terms "oligonucleotide", "siRNA", "siRNA oligonucleotide" and "siRNA's" are used interchangeably throughout the specification and include linear or circular oligomers of natural and/or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, substituted and alpha-anomeric forms thereof, peptide nucleic acids (PNA), locked nucleic acids (LNA), phosphorothioate, methylphosphonate, and the like. Oligonucleotides are capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like.

[00053] The oligonucleotide may be "chimeric", that is, composed of different regions. In the context of this invention "chimeric" compounds are oligonucleotides, which contain two or more chemical regions, for example, DNA region(s), RNA region(s), PNA region(s) etc. Each chemical region is made up of at least one monomer unit, *i.e.*, a nucleotide in the

case of an oligonucleotide compound. These oligonucleotides typically comprise at least one region wherein the oligonucleotide is modified in order to exhibit one or more desired properties. The desired properties of the oligonucleotide include, but are not limited, for example, to increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. Different regions of the oligonucleotide may therefore have different properties. The chimeric oligonucleotides of the present invention can be formed as mixed structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide analogs as described above.

[00054] The oligonucleotide can be composed of regions that can be linked in "register", that is, when the monomers are linked consecutively, as in native DNA, or linked via spacers. The spacers are intended to constitute a covalent "bridge" between the regions and have in preferred cases a length not exceeding about 100 carbon atoms. The spacers may carry different functionalities, for example, having positive or negative charge, carry special nucleic acid binding properties (intercalators, groove binders, toxins, fluorophors etc.), being lipophilic, inducing special secondary structures like, for example, alanine containing peptides that induce alpha-helices.

[00055] As used herein, the term "monomers" typically indicates monomers linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., from about 3-4, to about several hundreds of monomeric units. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, methylphosphonates, phosphoroselenoate, phosphoramidate, and the like, as more fully described below.

[00056] In the present context, the terms "nucleobase" covers naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "non-naturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanin, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof.

Especially interesting nucleobases are adenine, guanine, thymine, cytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

[00057] As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g., as described in Kornberg and Baker, *DNA Replication*, 2nd Ed. (Freeman, San Francisco, 1992).

[00058] "Analog" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., described generally by Scheit, *Nucleotide Analogs*, John Wiley, New York, 1980; Freier & Altmann, *Nucl. Acid. Res.*, 1997, 25(22), 4429-4443, Toulmé, J.J., *Nature Biotechnology* 19:17-18 (2001); Manoharan M., *Biochemica et Biophysica Acta* 1489:117-139(1999); Freier S., M., *Nucleic Acid Research*, 25:4429-4443 (1997), Uhlman, E., *Drug Discovery & Development*, 3: 203-213 (2000), Herdewin P., *Antisense & Nucleic Acid Drug Dev.*, 10:297-310 (2000),); 2'-O, 3'-C-linked [3.2.0] bicycloarabinonucleosides (see e.g. N.K. Christensen, et al, *J. Am. Chem. Soc.*, 120: 5458-5463 (1998). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g., duplex or triplex stability, specificity, or the like.

[00059] The term "stability" in reference to duplex or triplex formation generally designates how tightly an antisense oligonucleotide binds to its intended target sequence; more particularly, "stability" designates the free energy of formation of the duplex or triplex under physiological conditions. Melting temperature under a standard set of conditions, e.g., as described below, is a convenient measure of duplex and/or triplex stability. Preferably, oligonucleotides of the invention are selected that have melting temperatures of at least 45°C when measured in 100 mM NaCl, 0.1 mM EDTA and 10 mM phosphate buffer aqueous solution, pH 7.0 at a strand concentration of both the oligonucleotide and the target nucleic acid of 1.5 μ M. Thus, when used under physiological conditions, duplex or triplex formation will be substantially favored over the state in which the antigen and its target are dissociated. It is understood that a stable duplex or triplex may in some embodiments include mismatches between base pairs and/or among base triplets in the case of triplexes. Preferably, modified oligonucleotides, e.g. comprising LNA units, of the invention form perfectly matched duplexes and/or triplexes with their target nucleic acids.

[00060] As used herein, the term "downstream" when used in reference to a direction along a nucleotide sequence means in the direction from the 5' to the 3' end. Similarly, the term "upstream" means in the direction from the 3' to the 5' end.

[00061] As used herein, the term "gene" means the gene and all currently known variants thereof and any further variants which may be elucidated.

[00062] As used herein, "variant" of polypeptides refers to an amino acid sequence that is altered by one or more amino acid residues. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological activity may be found using computer programs well known in the art, for example, LASERGENE software (DNASTAR).

[00063] The term "variant," when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to a wild type gene. This definition may also include, for example, "allelic," "splice," "species," or "polymorphic" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. Of particular utility in the invention are variants of wild type target gene products. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

[00064] The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs,) or single base mutations in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population with a propensity for a disease state, that is susceptibility versus resistance.

[00065] As used herein, the term "mRNA" means the presently known mRNA transcript(s) of a targeted gene, and any further transcripts which may be elucidated.

[00066] By "desired RNA" molecule is meant any foreign RNA molecule which is useful from a therapeutic, diagnostic, or other viewpoint. Such molecules include antisense RNA molecules, decoy RNA molecules, enzymatic RNA, therapeutic editing RNA (Woolf and Stinchcomb, "Oligomer directed *In situ* reversion (ISR) of genetic mutations", filed Jul. 6, 1994, U.S. Ser. No. 08/271,280, hereby incorporated by reference) and agonist and antagonist RNA.

[00067] By "antisense RNA" is meant a non-enzymatic RNA molecule that binds to another RNA (target RNA) by means of RNA-RNA interactions and alters the activity of the target RNA (Eguchi et al., 1991 *Annu. Rev. Biochem.* 60, 631-652). By "enzymatic RNA" is meant an RNA molecule with enzymatic activity (Cech, 1988 *J. American. Med. Assoc.* 260, 3030-3035). Enzymatic nucleic acids (ribozymes) act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA.

[00068] By "decoy RNA" is meant an RNA molecule that mimics the natural binding domain for a ligand. The decoy RNA therefore competes with natural binding target for the binding of a specific ligand. For example, it has been shown that over-expression of HIV trans-activation response (TAR) RNA can act as a "decoy" and efficiently binds HIV tat protein, thereby preventing it from binding to TAR sequences encoded in the HIV RNA (Sullenger et al., 1990, *Cell*, 63, 601-608). This is meant to be a specific example. Those in the art will recognize that this is but one example, and other embodiments can be readily generated using techniques generally known in the art.

[00069] The term, "complementary" means that two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence. Normally, the complementary sequence of the oligonucleotide has at least 80% or 90%, preferably 95%, most preferably 100%, complementarity to a defined sequence. Preferably, alleles or variants thereof can be identified. A BLAST program also can be employed to assess such sequence identity.

[00070] The term "complementary sequence" as it refers to a polynucleotide sequence, relates to the base sequence in another nucleic acid molecule by the base-pairing rules. More particularly, the term or like term refers to the hybridization or base pairing between

nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 95% of the nucleotides of the other strand, usually at least about 98%, and more preferably from about 99 % to about 100%. Complementary polynucleotide sequences can be identified by a variety of approaches including use of well-known computer algorithms and software, for example the BLAST program.

[00071] As used herein, a "pharmaceutically acceptable" component is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

[00072] As used herein, the term "safe and effective amount" refers to the quantity of a component which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. By "therapeutically effective amount" is meant an amount of a compound of the present invention effective to yield the desired therapeutic response. For example, an amount effective to delay the growth of or to cause a cancer, either a sarcoma or lymphoma, or to shrink the cancer or prevent metastasis. The specific safe and effective amount or therapeutically effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

[00073] As used herein, a "pharmaceutical salt" include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids. Preferably the salts are made using an organic or inorganic acid. These preferred acid salts are chlorides, bromides, sulfates, nitrates, phosphates, sulfonates, formates, tartrates, maleates, malates, citrates, benzoates, salicylates, ascorbates, and the like. The most preferred salt is the hydrochloride salt.

[00074] As used herein, "cancer" refers to all types of cancer or neoplasm or malignant tumors found in mammals, including, but not limited to: leukemias, lymphomas, melanomas, carcinomas and sarcomas. Examples of cancers are cancer of the brain, breast, pancreas,

cervix, colon, head & neck, kidney, lung, non-small cell lung, melanoma, mesothelioma, ovary, sarcoma, stomach, uterus and Medulloblastoma.

[00075] The term "leukemia" refers broadly to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Leukemia is generally clinically classified on the basis of (1) the duration and character of the disease-acute or chronic; (2) the type of cell involved; myeloid (myelogenous), lymphoid (lymphogenous), or monocytic; and (3) the increase or non-increase in the number of abnormal cells in the blood-leukemic or aleukemic (subleukemic). Accordingly, the present invention includes a method of treating leukemia, and, preferably, a method of treating acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemetic leukemia, basophylic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia.

[00076] The term "sarcoma" generally refers to a tumor which is made up of a substance like the embryonic connective tissue and is generally composed of closely packed cells embedded in a fibrillar or homogeneous substance. Examples of sarcomas which can be treated with siRNA's and optionally a potentiator and/or chemotherapeutic agent include, but not limited to a chondrosarcoma, fibrosarcoma, lymphosarcoma, melanomasarcoma, myxosarcoma, osteosarcoma, Abemethy's sarcoma, adipose sarcoma, liposarcoma, alveolar soft part sarcoma, ameloblastic sarcoma, botryoid sarcoma, chloroma sarcoma, chorio carcinoma, embryonal sarcoma, Wilms' tumor sarcoma, endometrial sarcoma, stromal sarcoma, Ewing's sarcoma, fascial sarcoma, fibroblastic sarcoma, giant cell sarcoma, granulocytic sarcoma, Hodgkin's sarcoma, idiopathic multiple pigmented hemorrhagic sarcoma, immunoblastic sarcoma of B cells, lymphoma, immunoblastic sarcoma of T-cells,

Jensen's sarcoma, Kaposi's sarcoma, Kupffer cell sarcoma, angiosarcoma, leukosarcoma, malignant mesenchymoma sarcoma, parosteal sarcoma, reticulocytic sarcoma, Rous sarcoma, serocystic sarcoma, synovial sarcoma, and telangiectatic sarcoma.

[00077] The term "melanoma" is taken to mean a tumor arising from the melanocytic system of the skin and other organs. Melanomas which can be treated with siRNA's and optionally a potentiator and/or another chemotherapeutic agent include but not limited to, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, Cloudman's melanoma, S91 melanoma, Harding-Passey melanoma, juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungal melanoma, and superficial spreading melanoma.

[00078] The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Carcinomas which can be treated with siRNA's and optionally a potentiator and/or a chemotherapeutic agent include but not limited to, for example, acinar carcinoma, acinous carcinoma, adenocystic carcinoma, adenoid cystic carcinoma, carcinoma adenomatosum, carcinoma of adrenal cortex, alveolar carcinoma, alveolar cell carcinoma, basal cell carcinoma, carcinoma basocellulare, basaloid carcinoma, basosquamous cell carcinoma, bronchioalveolar carcinoma, bronchiolar carcinoma, bronchogenic carcinoma, cerebriform carcinoma, cholangiocellular carcinoma, chorionic carcinoma, colloid carcinoma, comedo carcinoma, corpus carcinoma, cribriform carcinoma, carcinoma en cuirasse, carcinoma cutaneum, cylindrical carcinoma, cylindrical cell carcinoma, duct carcinoma, carcinoma durum, embryonal carcinoma, encephaloid carcinoma, epiermoid carcinoma, carcinoma epitheliale adenoides, exophytic carcinoma, carcinoma ex ulcere, carcinoma fibrosum, gelatiniform carcinoma, gelatinous carcinoma, giant cell carcinoma, carcinoma gigantocellulare, glandular carcinoma, granulosa cell carcinoma, hair-matrix carcinoma, hematoid carcinoma, hepatocellular carcinoma, Hurthle cell carcinoma, hyaline carcinoma, hypemephroid carcinoma, infantile embryonal carcinoma, carcinoma in situ, intraepidermal carcinoma, intraepithelial carcinoma, Krompecher's carcinoma, Kulchitzky-cell carcinoma, large-cell carcinoma, lenticular carcinoma, carcinoma lenticulare, lipomatous carcinoma, lymphoepithelial carcinoma, carcinoma medullare, medullary carcinoma, melanotic carcinoma, carcinoma molle, mucinous carcinoma, carcinoma muciparum, carcinoma mucocellulare, mucoepidermoid carcinoma, carcinoma mucosum, mucous carcinoma, carcinoma myxomatodes, nasopharyngeal carcinoma, oat cell carcinoma, carcinoma ossificans, osteoid carcinoma, papillary carcinoma, periportal carcinoma, preinvasive

carcinoma, prickle cell carcinoma, pultaceous carcinoma, renal cell carcinoma of kidney, reserve cell carcinoma, carcinoma sarcomatodes, schneiderian carcinoma, scirrhous carcinoma, carcinoma scroti, signet-ring cell carcinoma, carcinoma simplex, small-cell carcinoma, solanoid carcinoma, spheroidal cell carcinoma, spindle cell carcinoma, carcinoma spongiosum, squamous carcinoma, squamous cell carcinoma, string carcinoma, carcinoma telangiectaticum, carcinoma telangiectodes, transitional cell carcinoma, carcinoma tuberosum, tuberous carcinoma, verrucous carcinoma, and carcinoma villosum.

[00079] Additional cancers which can be treated with siRNA's according to the invention include, for example, Hodgkin's Disease, Non-Hodgkin's Lymphoma, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, rhabdomyosarcoma, primary thrombocytosis, primary macroglobulinemia, small-cell lung tumors, primary brain tumors, stomach cancer, colon cancer, malignant pancreatic insulanoma, malignant carcinoid, urinary bladder cancer, premalignant skin lesions, testicular cancer, lymphomas, thyroid cancer, neuroblastoma, esophageal cancer, genitourinary tract cancer, malignant hypercalcemia, cervical cancer, endometrial cancer, adrenal cortical cancer, and prostate cancer.

[00080] A "heterologous" component refers to a component that is introduced into or produced within a different entity from that in which it is naturally located. For example, a polynucleotide derived from one organism and introduced by genetic engineering techniques into a different organism is a heterologous polynucleotide which, if expressed, can encode a heterologous polypeptide. Similarly, a promoter or enhancer that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous promoter or enhancer.

[00081] A "promoter," as used herein, refers to a polynucleotide sequence that controls transcription of a gene or coding sequence to which it is operably linked. A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources, are well known in the art and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources).

[00082] An "enhancer," as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. A large number of enhancers, from a variety of different sources are well known in the art and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides

comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences.

[00083] "Operably linked" refers to a juxtaposition, wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter is operably linked to a coding sequence if the promoter controls transcription of the coding sequence. Although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within or downstream of coding sequences. A polyadenylation sequence is operably linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence.

[00084] A "replicon" refers to a polynucleotide comprising an origin of replication which allows for replication of the polynucleotide in an appropriate host cell. Examples include replicons of a target cell into which a heterologous nucleic acid might be integrated (e.g., nuclear and mitochondrial chromosomes), as well as extrachromosomal replicons (such as replicating plasmids and episomes).

[00085] "Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene products") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of gene products to mammalian cells, as is known in the art and described herein.

[00086] "*In vivo*" gene delivery, gene transfer, gene therapy and the like as used herein, are terms referring to the introduction of a vector comprising an exogenous polynucleotide

directly into the body of an organism, such as a human or non-human mammal, whereby the exogenous polynucleotide is introduced to a cell of such organism *in vivo*.

[00087] A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a nucleic acid into a cell. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector which encodes a nucleic acid capable of stable replication and expression in a cell once the nucleic acid is transduced into the cell.

[00088] As used herein, a "target cell" or "recipient cell" refers to an individual cell or cell which is desired to be, or has been, a recipient of exogenous nucleic acid molecules, polynucleotides and/or proteins. The term is also intended to include progeny of a single cell.

[00089] A "vector" (sometimes referred to as gene delivery or gene transfer "vehicle") refers to a macromolecule or complex of molecules comprising a polynucleotide to be delivered to a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered may comprise a coding sequence of interest in gene therapy. Vectors include, for example, viral vectors (such as adenoviruses ("Ad"), adeno-associated viruses (AAV), and retroviruses), liposomes and other lipid-containing complexes, and other macromolecular complexes capable of mediating delivery of a polynucleotide to a host cell. Vectors can also comprise other components or functionalities that further modulate gene delivery and/or gene expression, or that otherwise provide beneficial properties to the targeted cells. As described and illustrated in more detail below, such other components include, for example, components that influence binding or targeting to cells (including components that mediate cell-type or tissue-specific binding); components that influence uptake of the vector nucleic acid by the cell; components that influence localization of the polynucleotide within the cell after uptake (such as agents mediating nuclear localization); and components that influence expression of the polynucleotide. Such components also might include markers, such as detectable and/or selectable markers that can be used to detect or select for cells that have taken up and are expressing the nucleic acid delivered by the vector. Such components can be provided as a natural feature of the vector (such as the use of certain viral vectors which have components or functionalities mediating binding and uptake), or vectors can be

modified to provide such functionalities. Other vectors include those described by Chen et al; *BioTechniques*, 34: 167-171 (2003). A large variety of such vectors are known in the art and are generally available.

[00090] A "recombinant viral vector" refers to a viral vector comprising one or more heterologous gene products or sequences. Since many viral vectors exhibit size-constraints associated with packaging, the heterologous gene products or sequences are typically introduced by replacing one or more portions of the viral genome. Such viruses may become replication-defective, requiring the deleted function(s) to be provided in trans during viral replication and encapsidation (by using, e.g., a helper virus or a packaging cell line carrying gene products necessary for replication and/or encapsidation). Modified viral vectors in which a polynucleotide to be delivered is carried on the outside of the viral particle have also been described (see, e.g., Curiel, D T, et al. *PNAS* 88: 8850-8854, 1991).

[00091] Viral "packaging" as used herein refers to a series of intracellular events that results in the synthesis and assembly of a viral vector. Packaging typically involves the replication of the "pro-viral genome", or a recombinant pro-vector typically referred to as a "vector plasmid" (which is a recombinant polynucleotide than can be packaged in an manner analogous to a viral genome, typically as a result of being flanked by appropriate viral "packaging sequences"), followed by encapsidation or other coating of the nucleic acid. Thus, when a suitable vector plasmid is introduced into a packaging cell line under appropriate conditions, it can be replicated and assembled into a viral particle. Viral "*rep*" and "*cap*" gene products, found in many viral genomes, are gene products encoding replication and encapsidation proteins, respectively. A "replication-defective" or "replication-incompetent" viral vector refers to a viral vector in which one or more functions necessary for replication and/or packaging are missing or altered, rendering the viral vector incapable of initiating viral replication following uptake by a host cell. To produce stocks of such replication-defective viral vectors, the virus or pro-viral nucleic acid can be introduced into a "packaging cell line" that has been modified to contain gene products encoding the missing functions which can be supplied in trans). For example, such packaging gene products can be stably integrated into a replicon of the packaging cell line or they can be introduced by transfection with a "packaging plasmid" or helper virus carrying gene products encoding the missing functions.

[00092] A "detectable marker gene" is a gene that allows cells carrying the gene to be specifically detected (e.g., distinguished from cells which do not carry the marker gene). A large variety of such marker gene products are known in the art. Preferred examples thereof

include detectable marker gene products which encode proteins appearing on cellular surfaces, thereby facilitating simplified and rapid detection and/or cellular sorting. By way of illustration, the *lacZ* gene encoding beta-galactosidase can be used as a detectable marker, allowing cells transduced with a vector carrying the *lacZ* gene to be detected by staining.

[00093] A "selectable marker gene" is a gene that allows cells carrying the gene to be specifically selected for or against, in the presence of a corresponding selective agent. By way of illustration, an antibiotic resistance gene can be used as a positive selectable marker gene that allows a host cell to be positively selected for in the presence of the corresponding antibiotic. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow selection for cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. A variety of such marker gene products have been described, including bifunctional (i.e. positive/negative) markers (see, e.g., WO 92/08796, published May 29, 1992, and WO 94/28143, published Dec. 8, 1994). Such marker gene products can provide an added measure of control that can be advantageous in gene therapy contexts.

[00094] "Diagnostic" or "diagnosed" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[00095] The terms "patient" or "individual" are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

[00096] "Treatment" is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. "Treatment" may also be specified as palliative care. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In tumor (e.g.,

cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy.

[00097] The treatment of neoplastic disease or neoplastic cells, refers to an amount of the vectors and/or peptides, described throughout the specification and in the Examples which follow, capable of invoking one or more of the following effects: (1) inhibition, to some extent, of tumor growth, including, (i) slowing down and (ii) complete growth arrest; (2) reduction in the number of tumor cells; (3) maintaining tumor size; (4) reduction in tumor size; (5) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention of tumor cell infiltration into peripheral organs; (6) inhibition, including (i) reduction, (ii) slowing down or (iii) complete prevention of metastasis; (7) enhancement of anti-tumor immune response, which may result in (i) maintaining tumor size, (ii) reducing tumor size, (iii) slowing the growth of a tumor, (iv) reducing, slowing or preventing invasion or (v) reducing, slowing or preventing metastasis; and/or (8) relief, to some extent, of one or more symptoms associated with the disorder.

[00098] Treatment of an individual suffering from an infectious disease organism refers to a decrease and elimination of the disease organism from an individual. For example, a decrease of viral particles as measured by plaque forming units or other automated diagnostic methods such as ELISA etc.

[00099] As used herein, a "pharmaceutically acceptable" component is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

Inhibition of Gene Expression

[000100] Enzymatic nucleic acid molecules (e.g., ribozymes) are nucleic acid molecules capable of catalyzing one or more of a variety of reactions, including the ability to repeatedly cleave other separate nucleic acid molecules in a nucleotide base sequence-specific manner. Such enzymatic nucleic acid molecules can be used, for example, to target virtually any RNA transcript (Zaug et al., 324, *Nature* 429 1986; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989).

[000101] Because of their sequence-specificity, trans-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecules can be designed to cleave specific RNA

targets within the background of cellular RNA. Such a cleavage event renders the mRNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

[000102] In general, enzymatic nucleic acids with RNA cleaving activity act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[000103] Several approaches such as *in vitro* selection (evolution) strategies (Orgel, 1979, Proc. R. Soc. London, B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce, 1989, *Gene*, 82, 83-87; Beaudry et al., 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker et al., 1994, *TIBTECH* 12, 268; Bartel et al., 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar et al., 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442).

[000104] The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The hammerhead ribozyme, for example, functions with a catalytic rate (k_{cat}) of about 1 min^{-1} in the presence of saturating (10 mM) concentrations of Mg^{2+} cofactor. An artificial "RNA ligase" ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of about 100 min^{-1} . In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min^{-1} . Finally, replacement of a specific residue within the catalytic core of the hammerhead with certain nucleotide analogues gives modified ribozymes that show as much as a 10-fold improvement in catalytic rate. These findings demonstrate that ribozymes can promote chemical transformations with catalytic rates that are significantly greater than those displayed *in vitro* by most natural self-cleaving ribozymes. It is then possible that the structures of certain self-cleaving ribozymes may be optimized to give maximal catalytic

activity, or that entirely new RNA motifs can be made that display significantly faster rates for RNA phosphodiester cleavage.

[000105] Intermolecular cleavage of an RNA substrate by an RNA catalyst that fits the "hammerhead" model was first shown in 1987 (Uhlenbeck, O. C. (1987) *Nature*, 328: 596-600). The RNA catalyst was recovered and reacted with multiple RNA molecules, demonstrating that it was truly catalytic.

[000106] Catalytic RNAs designed based on the "hammerhead" motif have been used to cleave specific target sequences by making appropriate base changes in the catalytic RNA to maintain necessary base pairing with the target sequences (Haseloff and Gerlach, *Nature*, 334, 585 (1988); Walbot and Bruening, *Nature*, 334, 196 (1988); Uhlenbeck, O. C. (1987) *Nature*, 328: 596-600; Koizumi, M., Iwai, S. and Ohtsuka, E. (1988) *FEBS Lett.*, 228: 228-230). This has allowed use of the catalytic RNA to cleave specific target sequences and indicates that catalytic RNAs designed according to the "hammerhead" model may possibly cleave specific substrate RNAs *in vivo*. (see Haseloff and Gerlach, *Nature*, 334, 585 (1988); Walbot and Bruening, *Nature*, 334, 196 (1988); Uhlenbeck, O. C. (1987) *Nature*, 328: 596-600).

[000107] RNA interference (RNAi) has become a powerful tool for blocking gene expression in mammals and mammalian cells. This approach requires the delivery of small interfering RNA (siRNA) either as RNA itself or as DNA, using an expression plasmid or virus and the coding sequence for small hairpin RNAs that are processed to siRNAs. Current expression systems for the production of siRNA *in vivo* rely on RNA polymerase III promoters. These are difficult to regulate and leave most of the RNA in the nucleus of the cell where it is inactive for RNA interference. This invention provides a DNA cassette for the cloning of small hairpin sequences which permit their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and permit the use of regulated and tissue specific promoters for gene expression.

[000108] As an illustrative example which is not meant to limit or construe the invention in any way, the following example is provided. This invention provides a DNA cassette for the cloning of small hairpin sequences which permits their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and will permit the use of regulated and tissue specific promoters for gene expression. The siRNA cassette comprises hammerhead and hairpin ribozymes flanking the cloning sites for hairpin RNA of variable sequence, depending on the

target of RNA interference. Figure 1 shows a hairpin RNA, here shown in red (GFP RNAi), is processed out through the autocatalytic activities of the ribozymes which cleave at the sites indicated by the arrows. These leave 6 extra nucleotides at the 5' end of the hairpin and 9 extra at the 3' end. The data indicate that long stretches of extra nucleotides can decrease the potency of small hairpin RNAs in RNA interference, but lengths less than 10 are permissible. The cloning site for insertion of the hairpin RNA can deviate from the prototype shown in this illustrative example, as long as internal base-pairing is preserved in the flanking hammerhead ribozyme (Rz) and hairpin ribozyme. This cassette was cloned in an AAV (Adeno-associated virus) vector in conjunction with the chicken beta actin promoter and the CMV immediate early enhancer to create pT-RRR. Digestion with HindIII and SpeI can be used to insert the genes for hairpin RNAs for delivery by recombinant AAV. The illustrative vector expressing the hairpin RNA is shown in Figure 2. Transfection of this plasmid plus a plasmid expressing GFP in 293 cells led to a 3-fold reduction in the number of cells expressing GFP at 48 hours post transfection and a 60% reduction at 72 hours post transfection based on FACS analysis, suggesting that this plasmid can lead to the production of functional siRNA in cultured cells.

[000109] In a preferred embodiment, the invention provides methods for treating cells comprising an infectious agent. Such treatment methods comprise administering a ribozyme-siRNA oligonucleotide to cells that comprise an oligonucleotide sequence of an infectious agent. The oligonucleotides preferably will be complementary to the infectious agent oligonucleotide sequence. A variety of cells may be treated in accordance with the compositions and methods of the invention, and typically mammalian cells are treated, especially primate cells such as human cells.

[000110] Inhibition of gene expression may be quantified by measuring either the endogenous target RNA or the protein produced by translation of the target RNA. Techniques for quantifying RNA and proteins are well known to one of ordinary skill in the art. In certain preferred embodiments, gene expression is inhibited by at least 10%, preferably by at least 33%, more preferably by at least 50%, and yet more preferably by at least 80%. In particularly preferred embodiments, of the invention gene expression is inhibited by at least 90%, more preferably by at least 95%, or by at least 99% up to 100% within cells in the organism. In preferred embodiments of the invention inhibition occurs rapidly after the organism comes into contact with the virus. In preferred embodiments significant inhibition of gene expression occurs within 24 hours after the subject comes into contact with the virus. In more preferred embodiments significant inhibition occurs within 12

hours after the subject comes into contact with the virus. In yet more preferred embodiments significant inhibition occurs between about 6 to 12 hours after the subject comes into contact with the virus. In yet more preferred embodiments significant inhibition occurs within less than about 6 hours after the subject comes into contact with the virus. By significant inhibition is meant sufficient inhibition to result in a detectable phenotype (e.g., inhibition of viral replication etc.) or a detectable decrease in RNA and/or protein corresponding to the gene being inhibited. Note that although in certain embodiments of the invention inhibition occurs in substantially all cells of the subject, in other preferred embodiments inhibition occurs in only a subset of cells expressing the heterologous gene.

[000111] In order to achieve inhibition of a target gene selectively within a given subject which it is desired to control, an RNAi preferably exhibits a high degree of sequence identity with corresponding segments in the subject. Preferably the degree of identity is more than about 80%. Untranslated regions (UTRs), i.e., 5' and 3' UTRs, frequently display a low degree of conservation across species since they are not constrained by the necessity of coding for a functional protein. Thus, in certain preferred embodiments the gene portion comprises or includes a UTR. If it is desired to inhibit a target gene within a number of different species which it is desired to control, the RNAi preferably exhibits a high degree of identity with the corresponding segments in these species and a low degree of identity with corresponding nucleic acid sequences in other species, particularly in mammals.

[000112] Selection of appropriate RNAi is facilitated by using computer programs that automatically align nucleic acid sequences and indicate regions of identity or homology. Such programs are used to compare nucleic acid sequences obtained, for example, by searching databases such as GenBank or by sequencing PCR products. Comparison of nucleic acid sequences from a range of species allows the selection of nucleic acid sequences that display an appropriate degree of identity between species. In the case of genes that have not been sequenced, Southern blots are performed to allow a determination of the degree of identity between genes in target species and other species. By performing Southern blots at varying degrees of stringency, as is well known in the art, it is possible to obtain an approximate measure of identity. These procedures allow the selection of RNAi that exhibit a high degree of complementarity to target nucleic acid sequences in a subject to be controlled and a lower degree of complementarity to corresponding nucleic acid sequences in other species. One skilled in the art will realize that there is considerable latitude in selecting appropriate regions of genes for use in the present invention.

[000113] In a preferred embodiment, small interfering RNA (siRNA) either as RNA itself or as DNA, is delivered to a cell using an expression plasmid or virus and the coding sequence for small hairpin RNAs that are processed to siRNAs.

[000114] In another preferred embodiment, a DNA cassette for the cloning of small hairpin sequences permit their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and permit the use of regulated and tissue specific promoters for gene expression.

[000115] In another preferred embodiment, cassette comprises hammerhead and hairpin ribozymes flanking the cloning sites for hairpin RNA of variable sequence, depending on the target of RNA interference. The hairpin RNA is processed out through the autocatalytic activities of the ribozymes which cleave at the sites indicated by the arrows. See for example, figure 1. These leave 6 extra nucleotides at the 5' end of the hairpin and 9 extra at the 3' end. Preferably, the stretches of extra nucleotides are about 2 to 40 nucleotides in length, preferably about 5 nucleotides in length.

[000116] In another preferred embodiment, cloning site for insertion of the hairpin RNA into an expression vector can be in any nucleotide location, as internal base-pairing is preserved in the flanking hammerhead ribozyme (Rz) and hairpin ribozyme.

[000117] In accordance with the invention target cells are selectively targeted by an siRNA based on their genetic makeup. Infectious disease almost invariably results in the acquisition of foreign nucleic acids, which could be targeted using this technology. Specific targets could be viral, e.g. HIV (virus or provirus) or bacterial, e.g. multi-drug resistant bacteria e.g. TB, fungal or protozoan. This technology can be especially useful in treating infections for which there is no effective anti-microbial or anti-viral agent (e.g. Ebola virus, etc.), or known or novel bio-terrorist agents.

[000118] Preferred siRNA's of the invention will hybridize (bind) to a target sequence, particularly a target oligonucleotide of an infectious agent such as a viral, bacterial, fungal or protozoan agent including those agents and sequences disclosed herein, under stringency conditions as may be assessed *in vitro*. Such conditions are disclosed and defined below.

[000119] The invention may be used against protein coding gene products as well as non-protein coding gene products. Examples of non-protein coding gene products include gene products that encode ribosomal RNAs, transfer RNAs, small nuclear RNAs, small cytoplasmic RNAs, telomerase RNA, RNA molecules involved in DNA replication, chromosomal rearrangement and the like. In another use for the invention, the siRNA delivery system can be used to target wild-type genes to provide tools for functional genetics

or to create cell-based and animal models of genetic disease, such as for example, target validation. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate disease animal models. In addition, cells from humans may be used. These systems may be used in a variety of applications. Such assays may be utilized as part of screening strategies designed to identify agents, such as compounds that are capable of ameliorating disease symptoms. Thus, the animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions that may be effective in treating disease and also to understand the mechanics behind diseases.

[000120] Cell-based systems may be used to identify compounds that may act to ameliorate disease symptoms. For example, such cell systems may be exposed to a compound suspected of exhibiting an ability to ameliorate disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed cells. After exposure, the cells are examined to determine whether one or more of the disease cellular phenotypes has been altered to resemble a more normal or more wild type, non-disease phenotype.

[000121] In addition, animal-based disease systems, may be used to identify compounds capable of ameliorating disease symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies, and interventions that may be effective in treating a disease or other phenotypic characteristic of the animal. For example, animal models may be exposed to a compound or agent suspected of exhibiting an ability to ameliorate disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of disease symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with the disease. Exposure may involve treating mother animals during gestation of the model animals described herein, thereby exposing embryos or fetuses to the compound or agent that may prevent or ameliorate the disease or phenotype. Neonatal, juvenile, and adult animals can also be exposed.

[000122] In another preferred embodiment, abnormal or cancer cells are targeted by the siRNAs. For example, many malignancies are associated with the presence of foreign DNA, e.g. Bcr-Abl, Bcl-2, HPV, and these provide unique molecular targets to permit selective malignant cell targeting. The approach can be used to target single base substitutions (e.g. K-ras, p53) or methylation changes. However, proliferation of cancer cells may also be caused by previously unexpressed gene products. These gene sequences can be targeted, thereby,

inhibiting further expression and ultimate death of the cancer cell. In other instances, transposons can be the cause of such deregulation and transposon sequences can be targeted, e.g. Tn5.

[000123] According to the present invention, an siRNA oligonucleotide is designed to be specific for a molecule, which either causes, participates in, or aggravates a disease state, in a patient. For example, in a viral infection, an siRNA can be targeted against molecules responsible for viral replication; a viral infection cycle, such as, for example, attachment to cellular ligands; viral gene products encoding host immune modulating functions.

Particularly preferred viral organisms causing human diseases according to the present invention include (but not restricted to) Filoviruses, Herpes viruses, Hepatitisviruses, Retroviruses, Orthomyxoviruses, Paramyxoviruses, Togaviruses, Picornaviruses, Papovaviruses and Gastroenteritisviruses. Other preferred, non-limiting examples of viral agents are listed in Table 1.

[000124] According to another preferred embodiment of the invention, the siRNA oligonucleotide is specific for human or domestic animal bacterial pathogens. Particularly preferred bacteria causing serious human diseases are the Gram positive organisms: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *E. faecium*, *Streptococcus pneumoniae* and the Gram negative organisms: *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Xanthomonas maltophilia*, *Escherichia coli*, *Enterobacter spp*, *Klebsiella pneumoniae* and *Salmonella spp*. The target molecules may include (but are not restricted to) molecules essential to bacterial survival and multiplication in the host organism, virulence gene products, gene products encoding single- or multi-drug resistance. However, gram negative bacteria are also within the scope of the invention.

[000125] In another preferred embodiment, the siRNA's are targeted to toxins produced by a disease agent such as anthrax. For example, anthrax which is one of the agents that can be used in a bioterrorist attack. Anthrax infection is mediated by spores of *Bacillus anthracis*, which can gain entry to the body through breaks in the skin, through inhalation, or through ingestion. Fatal anthrax is characterized by the establishment of a systemic bacteremia that is accompanied by an overwhelming toxemia. It seems that anthrax is a two-pronged attack with the bacteremia and/or toxemia contributing to the fatal syndrome of shock, hypoperfusion, and multiple organ system failure. The likelihood of developing systemic disease varies with the portal of organism entry, and is most pronounced for the inhalational route (reviewed in Dixon et al., 1999, *New England J. Med.* 341: 815-826). siRNA oligonucleotides can be targeted to the mRNAs that inhibit proliferation of the

bacteria in an infected patient and target the toxin producing gene products thereby eliminating the toxic effects of the anthrax infection. Alternatively, siRNA's could be targeted to any sequence target that is present in the organism and lacking in the host.

[000126] According to one preferred embodiment of the invention, the siRNA oligonucleotide is specific for protozoa infecting humans and causing human diseases. Particularly preferred protozoan organisms causing human diseases according to the present invention include (but not restricted to) Malaria e.g. *Plasmodium falciparum* and *M. ovale*, Trypanosomiasis (sleeping sickness) e.g. *Trypanosoma cruzi*, Leishmaniasis e.g. *Leishmania donovani*, Amebiasis e.g. *Entamoeba histolytica*.

[000127] According to one preferred embodiment of the invention, the siRNA oligonucleotide is specific for fungi causing pathogenic infections in humans. Particularly preferred fungi causing or associated with human diseases according to the present invention include (but not restricted to) *Candida albicans*, *Histoplasma neoformans*, *Coccidioides immitis* and *Penicillium marneffei*.

[000128] The invention in general provides a method for treating diseases, such as cancer and diseases which are caused by infectious agents such as viruses, bacteria, intra- and extra-cellular parasites, insertion elements, fungal infections, etc., which may also cause expression of gene products by a normally unexpressed gene, abnormal expression of a normally expressed gene or expression of an abnormal gene, comprising administering to a patient in need of such treatment an effective amount of an siRNA oligonucleotide; or a cocktail of different modified siRNA's; or a cocktail of different modified and unmodified siRNA oligonucleotides specific for the disease causing entity.

[000129] In accordance with the invention, siRNA oligonucleotide therapies comprise administered siRNA oligonucleotide which contacts (interacts with) the targeted mRNA from the gene, whereby expression of the gene is modulated, and expression is inhibited.. Such modulation of expression suitably can be a difference of at least about 10% or 20% relative to a control, more preferably at least about 30%, 40%, 50%, 60%, 70%, 80%, or 90% difference in expression relative to a control. It will be particularly preferred where interaction or contact with an siRNA oligonucleotide results in complete or essentially complete modulation of expression relative to a control, e.g., at least about a 95%, 97%, 98%, 99% or 100% inhibition of or increase in expression relative to control. A control sample for determination of such modulation can be comparable cells (*in vitro* or *in vivo*) that have not been contacted with the siRNA oligonucleotide.

[000130] The methods of the invention are preferably employed for treatment or prophylaxis against diseases caused abnormal cell growth and by infectious agents, particularly for treatment of infections as may occur in tissue such as lung, heart, liver, prostate, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, urinary tract or ovaries of a subject. The methods of the invention also may be employed to treat systemic conditions such as viremia or septicemia. The methods of the invention are also preferably employed for treatment of diseases and disorders associated with viral infections or bacterial infections, as well as any other disorder caused by an infectious agent.

[000131] Preferably, a disease agent is isolated from a patient and identified using diagnostic tools such as ELISA's RIAs, cell sorting, PCR and the like. However, a disease causing agent may be a novel agent to which siRNA oligonucleotides can be targeted. Sequencing data obtained from the agent can be used to construct an siRNA. Partial sequencing of the agent can be accomplished by any means known in the art. As an illustrative example which is not meant to limit or construe the invention in any way, the following is provided. The siRNA is designed to be complementary to selected sequences.

[000132] According to one preferred embodiment of the invention, the nucleobases in the siRNA may be modified to provided higher specificity and affinity for a target mRNA. For example nucleobases may be substituted with LNA monomers, which can be in contiguous stretches or in different positions. The modified siRNA, preferably has a higher association constant (K_a) for the target sequences than the complementary sequence. Binding of the modified or non-modified siRNA's to target sequences can be determined *in vitro* under a variety of stringency conditions using hybridization assays and as described in the examples which follow.

[000133] A fundamental property of oligonucleotides that underlies many of their potential therapeutic applications is their ability to recognize and hybridize specifically to complementary single stranded nucleic acids employing either Watson-Crick hydrogen bonding (A-T and G-C) or other hydrogen bonding schemes such as the Hoogsteen/reverse Hoogsteen mode. Affinity and specificity are properties commonly employed to characterize hybridization characteristics of a particular oligonucleotide. Affinity is a measure of the binding strength of the oligonucleotide to its complementary target (expressed as the thermostability (T_m) of the duplex). Each nucleobase pair in the duplex adds to the thermostability and thus affinity increases with increasing size (No. of nucleobases) of the oligonucleotide. Specificity is a measure of the ability of the oligonucleotide to discriminate between a fully complementary and a mismatched target sequence. In other words,

specificity is a measure of the loss of affinity associated with mismatched nucleobase pairs in the target.

[000134] The utility of an siRNA oligonucleotide for modulation (including inhibition) of an mRNA can be readily determined by simple testing. Thus, an *in vitro* or *in vivo* expression system comprising the targeted mRNA, mutations or fragments thereof, can be contacted with a particular siRNA oligonucleotide (modified or un modified) and levels of expression are compared to a control, that is, using the identical expression system which was not contacted with the siRNA oligonucleotide. This is described in detail in the examples which follow.

[000135] siRNA oligonucleotides may be used in combinations. For instance, a cocktail of several different siRNA modified and/or unmodified oligonucleotides, directed against different regions of the same gene, may be administered simultaneously or separately. For example, the vector expressing the ribozyme siRNA cassette may comprise one or a plurality of such cassettes in tandem, for example, 2, 3, 4 etc.

[000136] According to one preferred embodiment, the siRNA is specific for target oligonucleotides responsible for viral replication; viral infection cycle such as attachment to cellular ligands; viral gene products encoding host immune modulating functions. Examples of viral organisms include, but not restricted to, those listed in table 1. For information about the viral organisms see Fields of Virology, 3. ed., vol 1 and 2, BN Fields *et al.* (eds.). Non-limiting examples of targets of selected viral organisms are listed in table 2.

Table 1. Selected viral organisms causing human diseases

Herpesviruses	
	Alpha-herpesviruses:
	Herpes simplex virus 1 (HSV-1)
	Herpes simplex virus 2 (HSV-2)
	Varicella Zoster virus (VZV)
	Beta-herpesviruses:
	Cytomegalovirus (CMV)
	Herpes virus 6 (HHV-6)
	Gamma-herpesviruses:
	Epstein-Barr virus (EBV)
	Herpes virus 8 (HHV-8)
Hepatitis viruses	
	Hepatitis A virus
	Hepatitis B virus

	Hepatitis C virus
	Hepatitis D virus
	Hepatitis E virus
Retroviruses	
	Human Immunodeficiency 1 (HIV-1)
Orthomyxoviruses	
	Influenzaviruses A, B and C
Paramyxoviruses	
	Respiratory Syncytial virus (RSV)
	Parainfluenza viruses (PI)
	Mumps virus
	Measles virus
Togaviruses	
	Rubella virus
Picornaviruses	
	Enteroviruses
	Rhinoviruses
	Coronaviruses
Papovaviruses	
	Human papilloma viruses (HPV)
	Polyomaviruses (BKV and JCV)
Gastroenteritisviruses	
Filoviridae	
Bunyaviridae	
Rhabdoviridae	
Flaviviridae	

Table 2 Target gene products of viral organisms

Organism	target gene	open reading frame	gene product
HIV	gag:	MA	p17
		CA	p24
		NC	p7
	pol:		p6
		PR	p15
		RT	p66
	env:		p31
			gp120
			gp41
	tat		transcriptional transactivator
	rev		regulator of viral expression
	vif		
	vpr		
	vpu		
	nef		
RSV	NS1		
	NS2		
	L		
	2-5A-dependent RNase L		
HPV	E1		helicase
			transcription regulator
			late NS protein
			transforming protein
			transforming protein
			transforming protein
HCV	NS3		major capsid protein
			minor capsid protein
	NS3		protease
			helicase
	HCV-IRES		
	NS5B		
			polymerase
	HCMV DNA polymerase		
HCMV	DNA polymerase	IE1	
		IE2	
		UL36	
		UL37	
		UL44	polymerase asc. protein

	UL54	polymerase
	UL57	DNA binding protein
	UL70	primase
	UL102	primase asc. protein
	UL112	
	UL113	
	IRS1	
VZV	6	
	16	
	18	
	19	
	28	
	29	
	31	
	39	
	42	
	45	
	47	
	51	
	52	
	55	
	62	
	71	
HSV	IE4	US1
	IE5	US12
	IE110	ICP0
	IE175	ICP4
	UL5	helicase
	UL8	helicase
	UL13	capsid protein
	UL30	polymerase
	UL39	ICP6
	UL42	DNA binding protein

[000137] Information about the above selected gene products, open reading frames and gene products is found in the following references: Field A.K. and Biron, K.K. "The end of innocence" revisited: resistance of herpesviruses to antiviral drugs. *Clin. Microbiol. Rev.* 1994; 7: 1-13. Anonymous. Drug resistance in cytomegalovirus: current knowledge and implications for patient management. *J. Acquir. Immune Defic. Syndr. Hum. Retrovir.* 1996; 12: S1-SS22. Kelley R *et al.*. Varicella in children with perinatally acquired human

immunodeficiency virus infection. *J Pediatr* 1994; 124: 271-273. Hanecak et al. Antisense oligonucleotides inhibition of hepatitis C virus. gene expression in transformed hepatocytes. *J Virol* 1996; 70: 5203-12. Walker Drug discovery Today 1999; 4: 518-529. Zhang *et al.* Antisense oligonucleotides inhibition of hepatitis C virus (HCV) gene expression in livers of mice infected with an HCV-Vaccinia virus recombinant. *Antim. Agents Chemotherapy* 1999; 43, 347- 53. Feigin RD, Cherry JD, *eds.* Textbook of pediatric infectious diseases. Philadelphia: WB Saunders, 1981. Chen B.*et al.*, Induction of apoptosis of human cervical carcinoma cell line SiHa by antisense oligonucleotide og human papillomavirus type 16 E6 gene. 2000; 21(3): 335-339. The human herpesviruses. New York: Raven Press; 1993. DeClerque E, Walker RT, *eds.* Antiviral drug development: a multi-disciplinary approach. Plenum; 1987. Antiviral Drug Resistance (Richman, D.D., ed.), Wiley, Chichester, 1995. Flint SJ *et al.* *eds.* Principles of virology: Molecular biology, pathogene productsis and control.

[000138] It should be appreciated that in the above table 2, an indicated gene means the gene and all currently known variants thereof, including the different mRNA transcripts that the gene and its variants can give rise to, and any further gene variants which may be elucidated. In general, however, such variants will have significant sequence identity to a sequence of table 2 above, e.g. a variant will have at least about 70 percent sequence identity to a sequence of the above table 2, more typically at least about 75, 80, 85, 90, 95, 97, 98 or 99 percent sequence identity to a sequence of the above table 2. Sequence identity of a variant can be determined by any of a number of standard techniques such as a BLAST program <http://www.ncbi.nlm.nih.gov/blast/>).

[000139] Sequences for the gene products listed in Table 2 can be found in GenBank (<http://www.ncbi.nlm.nih.gov/>). The gene sequences may be genomic, cDNA or mRNA sequences. Preferred sequences are viral gene products containing the complete coding region and 5' untranslated sequences that are involved in viral replication.

[000140] *In vitro* propagation of virus causing human diseases: To screen for antiviral effect of siRNA oligonucleotides viral particles are propagated in *in vitro* culture systems of appropriate mammalian cells. Initial screening is typically performed in transformed cell lines. More thorough screening is typically performed in human diploid cells.

Table 3. Examples of *in vitro* propagation of viruses.

Organism	WI-38 or MRC-5	HeLa or HEp-2	PRMK or PCMK
HSV	C,D,S	D	D
HCMV	C,F	-	-
VZV	C,F	-	-
Adeno	D	D	D
RSV	S	S	S
Polio	D	D	D
Echo	D	-	D
Rhino	D,F	-	D,F

C is cytomegaly, D is cell destruction, F is marked focality, H is hemadsorption and S is formation of syncytium. "-" means that the cell line does not sustain growth of the virus. WI-38 is a human diploid fibroblast cell line. MRC-5 is human lung fibroblasts. HeLa is a human aneuploid epithelial cell line. PRMK is primary rhesus monkey kidney cells. PCMK is primary cynomolgus monkey kidney cells.

[000141] Likewise Vero cells (green monkey kidney cells) and Mewo cells will sustain the growth of for example herpesviruses. References: DeClerque E, Walker RT, eds.

Antiviral drug development: a multi-disciplinary approach. Plenum; 1987. Antiviral Drug Resistance (Richman, D.D., ed.), Wiley, Chichester, 1995. Cytomegalovirus protocols, J. Sinclair (ed.), Humana Press. HIV Protocols, N. Michael and JH Kim (eds.), Humana press. Hepatitis C Protocols, JYN Lau (ed.), Humana Press. Antiviral Methods and Protocols, D Kinchington and RF Schinazi, Humana Press.

[000142] Protozoan infections: According to one preferred embodiment of the invention, the siRNA oligonucleotide is specific for protozoan organisms infecting humans and causing human diseases. Such protozoa include, but are not restricted to, the following: 1. Malaria e.g. *Plasmodium falciparum* and *M. ovale*. (references: Malaria by M Wahlgren and P Perlman (eds.), Harwood Academic Publishers, 1999. Molecular Immunological Considerations in Malaria Vaccine Development by MF Good and AJ Saul, CRC Press 1993). 2. Trypanosomiasis (sleeping sickness) e.g. *Trypanosoma cruzi* (reference: Progress in Human African Trypanosomiasis, Sleeping Sickness by M Dumas *et al.* (eds.), Springer Verlag 1998). 3. Leishmaniasis e.g. *Leishmania donovani* (reference: AL Banuals *et al.*, Molecular Epidemiology and Evolutionary Genetics of *Leishmania* Parasites. *Int J Parasitol* 1999;29:1137-47). 4. Amebiasis e.g. *Entamoeba histolytica* (RP Stock *et al.*, Inhibition of Gene Expression in *Entamoeba histolytica* with Antisense Peptide Nucleic Acid Oligomers. *Nature Biotechnology* 2001;19:231-34).

[000143] Fungal infections: According to one preferred embodiment of the invention, the siRNA oligonucleotide is specific for fungi cause pathogenic infections in humans. These

include, but are not restricted to, the following: *Candida albicans* (references: AH Groll *et al.*, Clinical pharmacology of systemic antifungal agents: a comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. *Adv. Pharmacol.* 1998;44:343-501. MDD Backer *et al.*, An antisense-based functional genomics approach for identification of gene products critical for growth of *Candida albicans*. *Nature Biotechnology* 2001;19:235-241) and others, e.g., *Histoplasma neoformans*, *Coccidioides immitis* and *Penicillium marneffei* (reference: SA Marques *et al.*, Mycoses associated with AIDS in the Third World. *Med Mycol* 2000; 38 *Suppl.* 1:269-79).

[000144] Host cellular gene products involved in viral diseases: According to one preferred embodiment of the invention, the siRNA oligonucleotide is specific for host cellular gene products involved in viral diseases. Besides gene products encoded by viruses for their replication, the initial step to infection is binding to cellular ligands. For example CD4, chemokine receptors such as CCR3, CCR5 are required for HIV infection. Furthermore, viruses also upregulate certain chemokines which aid in their replication, for example in the case of HIV there is an increase in IL-2 which results in an increase of CD4⁺ T cells, allowing for an increase in the pool of cells for further infection in the early stages of the disease. The siRNA oligonucleotides may be used to prevent any further upregulation of gene products that may aid in the infectivity and replication rate of the viruses. Preferred targets are the 5' untranslated sequences of ligands used by viruses to infect a cell, or any other cellular factor that aids in the replication of the viruses. Particularly preferred are human cDNA sequences. According to the invention siRNA oligonucleotides may be used to modulate the expression of gene products (e.g. mRNA) involved in the viral infection cycle.

[000145] siRNA oligonucleotides against gene products involved in infectious diseases caused by viruses, bacteria, protozoa, fungi, parasites, etc., may be used in combinations. For instance, a cocktail of several different siRNA oligonucleotides, directed against different regions of the same gene, may be administered simultaneously or separately. Also, combinations of siRNA oligonucleotides specific for different gene products, such as for instance the HBV P, S, and C mRNA, may be administered simultaneously or separately. siRNA oligonucleotides may also be administered in combination with other antiviral drugs, antibiotics, etc.

[000146] In the practice of the present invention, target gene products may be single-stranded or double-stranded DNA or RNA. Short dsRNA can be used to block transcription if they are of the same sequence as the start site for transcription of a particular gene. See, for example, Janowski *et al.* *Nature Chemical Biology*, 2005, 10:1038. It is understood that the

target to which the siRNA oligonucleotides of the invention are directed include allelic forms of the targeted gene and the corresponding mRNAs including splice variants. There is substantial guidance in the literature for selecting particular sequences for siRNA oligonucleotides given a knowledge of the sequence of the target polynucleotide. Preferred mRNA targets include the 5' cap site, tRNA primer binding site, the initiation codon site, the mRNA donor splice site, and the mRNA acceptor splice site.

[000147] Where the target polynucleotide comprises a mRNA transcript, sequence complementary oligonucleotides can hybridize to any desired portion of the transcript. Such oligonucleotides are, in principle, effective for inhibiting translation, and capable of inducing the effects described herein. It is hypothesized that translation is most effectively inhibited by the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the 5'-region of mRNA transcript are preferred. Oligonucleotides complementary to the mRNA, including the initiation codon (the first codon at the 5' end of the translated portion of the transcript), or codons adjacent to the initiation codon, are preferred.

Chimeric/modified Ribozyme-siRNA Cassettes

[000148] In accordance with this invention, persons of ordinary skill in the art will understand that mRNA includes not only the coding region which carries the information to encode a protein using the three letter genetic code, including the translation start and stop codons, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region, intron regions and intron/exon or splice junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the coding ribonucleotides. In preferred embodiments, the oligonucleotide is targeted to a translation initiation site (AUG codon) or sequences in the coding region, 5' untranslated region or 3'-untranslated region of an mRNA. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing or maturation of the RNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to cause interference with protein expression.

[000149] Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras", in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of

at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. In one preferred embodiment, a chimeric oligonucleotide comprises at least one region modified to increase target binding affinity, and, usually, a region that acts as a substrate for RNase H. Affinity of an oligonucleotide for its target (in this case, a nucleic acid encoding ras) is routinely determined by measuring the T_m of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the T_m , the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is modified comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. In other preferred embodiments, RNA modifications include 2'-fluoro, 2'-amino and 2' O-methyl modifications on the ribose of pyrimidines, abasic residues or an inverted base at the 3' end of the RNA. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (i.e., higher target binding affinity) than; 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance RNAi oligonucleotide inhibition of gene expression. RNase H is a cellular endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of RNAi inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred embodiment, the chimeric oligonucleotide is also modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide.

Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. Oligonucleotides which contain at least one phosphorothioate modification are presently more preferred. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. Some desirable modifications can be found in De Mesmaeker *et al. Acc. Chem. Res.* 1995, 28:366-374.

[000150] Specific examples of some preferred oligonucleotides envisioned for this invention include those comprising modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly $\text{CH}_2\text{--NH--O--CH}_2$, $\text{CH}_2\text{--N(CH}_3\text{)--O--CH}_2$ [known as a methylene(methylimino) or MMI backbone], $\text{CH}_2\text{--O--N(CH}_3\text{)--CH}_2$, $\text{CH}_2\text{--N(CH}_3\text{)--N(CH}_3\text{)--CH}_2$ and $\text{O--N(CH}_3\text{)--CH}_2\text{--CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O--P--O--CH_2). The amide backbones disclosed by De Mesmaeker *et al. Acc. Chem. Res.* 1995, 28:366-374) are also preferred. Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al. Science* 1991, 254, 1497). Oligonucleotides may also comprise one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH, SH, SCH_3 , F, OCN, OCH_3 , OCH_3 , OCH_3 , $\text{O(CH}_2\text{)}_n\text{CH}_3$, $\text{O(CH}_2\text{)}_n\text{NH}_2$ or $\text{O(CH}_2\text{)}_n\text{CH}_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3 ; OCF_3 ; O--, S--, or N-alkyl; O--, S--, or N-alkenyl; SOCH_3 ; SO_2CH_3 ; ONO_2 ; NO_2 ; N_3 ; NH_2 ; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification

includes 2'-methoxyethoxy [2'-O-CH₂ CH₂ OCH₃, also known as 2'-O-(2-methoxyethyl)] (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O--CH₃), 2'-propoxy (2'-OCH₂ CH₂CH₃) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

[000151] Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-Me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2' deoxycytosine and often referred to in the art as 5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N₆ (6-aminohexyl)adenine and 2,6-diaminopurine. Kornberg, A., *DNA Replication*, W. H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al. *Nucl. Acids Res.* 1987, 15:4513). A "universal" base known in the art, e.g., inosine, may be included. 5-Me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

[000152] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, 86, 6553), cholic acid (Manoharan et al. *Bioorg. Med. Chem. Lett.* 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al. *Ann. N.Y. Acad. Sci.* 1992, 660, 306; Manoharan et al. *Bioorg. Med. Chem. Lett.* 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.* 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al. *EMBO J.* 1991, 10, 111; Kabanov et al. *FEBS Lett.* 1990, 259, 327; Svinarchuk et al. *Biochimie* 1993, 75, 49), a phospholipid,

e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651; Shea et al. *Nucl. Acids Res.* 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al. *Nucleosides & Nucleotides* 1995, 14, 969), or adamantane acetic acid (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255.

[000153] It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a single nucleoside within an oligonucleotide. The present invention also includes oligonucleotides which are chimeric oligonucleotides as hereinbefore defined.

[000154] In another embodiment, the nucleic acid molecule of the present invention is conjugated with another moiety including but not limited to abasic nucleotides, polyether, polyamine, polyamides, peptides, carbohydrates, lipid, or polyhydrocarbon compounds. Those skilled in the art will recognize that these molecules can be linked to one or more of any nucleotides comprising the nucleic acid molecule at several positions on the sugar, base or phosphate group.

[000155] The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of one of ordinary skill in the art. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling VA) to synthesize fluorescently labeled, biotinylated or other modified oligonucleotides such as cholesterol-modified oligonucleotides.

[000156] In accordance with the invention, use of modifications such as the use of LNA monomers to enhance the potency, specificity and duration of action and broaden the routes of administration of oligonucleotides comprised of current chemistries such as MOE, ANA, FANA, PS etc (ref: Recent advances in the medical chemistry of antisense oligonucleotide by Uhlman, *Current Opinions in Drug Discovery & Development* 2000 Vol 3 No 2). This can

be achieved by substituting some of the monomers in the current oligonucleotides by LNA monomers. The LNA modified oligonucleotide may have a size similar to the parent compound or may be larger or preferably smaller. It is preferred that such LNA-modified oligonucleotides contain less than about 70%, more preferably less than about 60%, most preferably less than about 50% LNA monomers and that their sizes are between about 10 and 25 nucleotides, more preferably between about 12 and 20 nucleotides.

Cancer Therapy

[000157] In another preferred embodiment, the siRNA oligonucleotides are used to treat patients susceptible to or suffering from cancer. Gene products which are over expressed in a cancer cell can be identified so that the siRNA oligonucleotide selectively targets the cancer cell as opposed to normal cells. For example, Expressed Sequenced Tags (ESTs), can be used to identify nucleic acid molecules which are over expressed in a cancer cell [expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, 2000, 480, 2-16; Larsson, *et al.*, *J. Biotechnology.*, 2000, 80, 143-57)]. ESTs from a variety of databases can be identified. For example, preferred databases include, for example, Online Mendelian Inheritance in Man (OMIM), the Cancer Genome Anatomy Project (CGAP), GenBank, EMBL, PIR, SWISS-PROT, and the like. OMIM, which is a database of genetic mutations associated with disease, was developed, in part, for the National Center for Biotechnology Information (NCBI). OMIM can be accessed through the world wide web of the Internet, at, for example, ncbi.nlm.nih.gov/Omim/. CGAP, which is an interdisciplinary program to establish the information and technological tools required to decipher the molecular anatomy of a cancer cell. CGAP can be accessed through the world wide web of the Internet, at, for example, ncbi.nlm.nih.gov/ncicgap/. Some of these databases may contain complete or partial nucleotide sequences. In addition, alternative transcript forms can also be selected from private genetic databases. Alternatively, nucleic acid molecules can be selected from available publications or can be determined especially for use in connection with the present invention.

[000158] Alternative transcript forms can be generated from individual ESTs which are within each of the databases by computer software which generates contiguous sequences. In another embodiment of the present invention, the nucleotide sequence of the target nucleic acid molecule is determined by assembling a plurality of overlapping ESTs. The EST database (dbEST), which is known and available to those skilled in the art, comprises approximately one million different human mRNA sequences comprising from about 500 to

1000 nucleotides, and various numbers of ESTs from a number of different organisms. dbEST can be accessed through the world wide web of the Internet, at, for example, ncbi.nlm.nih.gov/dbEST/index.html. These sequences are derived from a cloning strategy that uses cDNA expression clones for genome sequencing. ESTs have applications in the discovery of new gene products, mapping of genomes, and identification of coding regions in genomic sequences. Another important feature of EST sequence information that is becoming rapidly available is tissue-specific gene expression data. This can be extremely useful in targeting mRNA from selective gene(s) for therapeutic intervention. Since EST sequences are relatively short, they must be assembled in order to provide a complete sequence. Because every available clone is sequenced, it results in a number of overlapping regions being reported in the database. The end result is the elicitation of alternative transcript forms from, for example, normal cells and cancer cells.

[000159] Assembly of overlapping ESTs extended along both the 5' and 3' directions results in a full-length "virtual transcript." The resultant virtual transcript may represent an already characterized nucleic acid or may be a novel nucleic acid with no known biological function. The Institute for Genomic Research (TIGR) Human Genome Index (HGI) database, which is known and available to those skilled in the art, contains a list of human transcripts. TIGR can be accessed through the world wide web of the Internet, at, for example, tigr.org. Transcripts can be generated in this manner using TIGR-Assembler, an engine to build virtual transcripts and which is known and available to those skilled in the art. TIGR-Assembler is a tool for assembling large sets of overlapping sequence data such as ESTs, BACs, or small genomes, and can be used to assemble eukaryotic or prokaryotic sequences. TIGR-Assembler is described in, for example, Sutton, et al., *Genome Science & Tech.*, 1995, 1, 9-19, which is incorporated herein by reference, and can be accessed through the file transfer program of the Internet, at, for example, tigr.org/pub/software/TIGR. assembler. In addition, GLAXO-MRC, which is known and available to those skilled in the art, is another protocol for constructing virtual transcripts. Identification of ESTs and generation of contiguous ESTs to form full length RNA molecules is described in detail in U.S. application Ser. No. 09/076,440, which is incorporated herein by reference.

[000160] Gene products which are overexpressed by cancer cells as compared to normal cells, for example, gene products expressed at least 5 fold greater in pancreatic cancers compared to normal tissues can be identified. Gene expression can be analyzed by Serial Analysis of Gene Expression (SAGE), which is based on the identification of and characterization of partial, defined sequences of transcripts corresponding to gene segments

[SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, 2000, 5, 415-425)]. These defined transcript sequence "tags" are markers for gene products which are expressed in a cell, a tissue, or an extract, for example.

Identification of Target Nucleic acid Sequences

[000161] In a preferred embodiment, the compositions of the invention target desired nucleic acid sequences. Target nucleic acid sequences can be identified by a variety of methods such as SAGE. SAGE is based on several principles. First, a short nucleotide sequence tag (9 to 10 b.p.) contains sufficient information content to uniquely identify a transcript provided it is isolated from a defined position within the transcript. For example, a sequence as short as 9 b.p. can distinguish 262,144 transcripts given a random nucleotide distribution at the tag site, whereas estimates suggest that the human genome encodes about 80,000 to 200,000 transcripts (Fields, *et al.*, *Nature Genetics*, 7:345 1994). The size of the tag can be shorter for lower eukaryotes or prokaryotes, for example, where the number of transcripts encoded by the genome is lower. For example, a tag as short as 6-7 b.p. may be sufficient for distinguishing transcripts in yeast.

[000162] Second, random dimerization of tags allows a procedure for reducing bias (caused by amplification and/or cloning). Third, concatenation of these short sequence tags allows the efficient analysis of transcripts in a serial manner by sequencing multiple tags within a single vector or clone. As with serial communication by computers, wherein information is transmitted as a continuous string of data, serial analysis of the sequence tags requires a means to establish the register and boundaries of each tag. The concept of deriving a defined tag from a sequence in accordance with the present invention is useful in matching tags of samples to a sequence database. In the preferred embodiment, a computer method is used to match a sample sequence with known sequences.

[000163] The tags used herein, uniquely identify gene products. This is due to their length, and their specific location (3') in a gene from which they are drawn. The full length gene products can be identified by matching the tag to a gene data base member, or by using the tag sequences as probes to physically isolate previously unidentified gene products from cDNA libraries. The methods by which gene products are isolated from libraries using DNA probes are well known in the art. See, for example, Veculescu et al., *Science* 270: 484 (1995), and Sambrook et al. (1989), *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.). Once a gene or transcript has been identified, either by matching to a data base entry, or by physically hybridizing to a

cDNA molecule, the position of the hybridizing or matching region in the transcript can be determined. If the tag sequence is not in the 3' end, immediately adjacent to the restriction enzyme used to generate the SAGE tags, then a spurious match may have been made.

Confirmation of the identity of a SAGE tag can be made by comparing transcription levels of the tag to that of the identified gene in certain cell types.

[000164] Analysis of gene expression is not limited to the above methods but can include any method known in the art. All of these principles may be applied independently, in combination, or in combination with other known methods of sequence identification.

[000165] Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, 2000, 480, 17-24; Celis, et al., *FEBS Lett.*, 2000, 480, 2-16), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, 1999, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Jungblut, et al., *Electrophoresis*, 1999, 20, 2100-10), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, 2000, 286, 91-98; Larson, et al., *Cytometry*, 2000, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, 2000, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, 1998, 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, 1999, 35, 1895-904) and mass spectrometry methods (reviewed in (*Comb. Chem. High Throughput Screen*, 2000, 3, 235-41)).

[000166] In yet another aspect, siRNA oligonucleotides that selectively bind to variants of target gene expression products are useful for treatment of cancer. For example, p53 mutants are well known in a variety of tumors. A "variant" is an alternative form of a gene. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

[000167] Sequence similarity searches can be performed manually or by using several available computer programs known to those skilled in the art. Preferably, Blast and Smith-Waterman algorithms, which are available and known to those skilled in the art, and the like can be used. Blast is NCBI's sequence similarity search tool designed to support analysis of

nucleotide and protein sequence databases. Blast can be accessed through the world wide web of the Internet, at, for example, ncbi.nlm.nih.gov/BLAST/. The GCG Package provides a local version of Blast that can be used either with public domain databases or with any locally available searchable database. GCG Package v9.0 is a commercially available software package that contains over 100 interrelated software programs that enables analysis of sequences by editing, mapping, comparing and aligning them. Other programs included in the GCG Package include, for example, programs which facilitate RNA secondary structure predictions, nucleic acid fragment assembly, and evolutionary analysis. In addition, the most prominent genetic databases (GenBank, EMBL, PIR, and SWISS-PROT) are distributed along with the GCG Package and are fully accessible with the database searching and manipulation programs. GCG can be accessed through the Internet at, for example, <http://www.gcg.com/>. Fetch is a tool available in GCG that can get annotated GenBank records based on accession numbers and is similar to Entrez. Another sequence similarity search can be performed with GeneWorld and GeneThesaurus from Pangea. GeneWorld 2.5 is an automated, flexible, high-throughput application for analysis of polynucleotide and protein sequences. GeneWorld allows for automatic analysis and annotations of sequences. Like GCG, GeneWorld incorporates several tools for homology searching, gene finding, multiple sequence alignment, secondary structure prediction, and motif identification. GeneThesaurus 1.0™ is a sequence and annotation data subscription service providing information from multiple sources, providing a relational data model for public and local data.

[000168] Another alternative sequence similarity search can be performed, for example, by BlastParse. BlastParse is a PERL script running on a UNIX platform that automates the strategy described above. BlastParse takes a list of target accession numbers of interest and parses all the GenBank fields into "tab-delimited" text that can then be saved in a "relational database" format for easier search and analysis, which provides flexibility. The end result is a series of completely parsed GenBank records that can be easily sorted, filtered, and queried against, as well as an annotations-relational database.

[000169] In accordance with the invention, paralogs can be identified for designing the appropriate siRNA oligonucleotide. Paralogs are genes within a species that occur due to gene duplication, but have evolved new functions, and are also referred to as isotypes.

[000170] The polynucleotides of this invention can be isolated using the technique described in the experimental section or replicated using PCR. The PCR technology is the subject matter of U.S. Pat. Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and

described in PCR: The Polymerase Chain Reaction (Mullis et al. eds, Birkhauser Press, Boston (1994)) and references cited therein. Alternatively, one of skill in the art can use the identified sequences and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, nucleotides, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the polynucleotide into a suitable replication vector and insert the vector into a suitable host cell (prokaryotic or eukaryotic) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining polynucleotides by this method is further provided herein as well as the polynucleotides so obtained.

Disease Therapy

[000171] In another preferred embodiment, the siRNA can be used in treating diseases wherein immune cells are involved in the disease, such as autoimmune disease; hypersensitivity to allergens; organ rejection; inflammation; and the like. Examples of inflammation associated with conditions such as: adult respiratory distress syndrome (ARDS) or multiple organ injury syndromes secondary to septicemia or trauma; reperfusion injury of myocardial or other tissues; acute glomerulonephritis; reactive arthritis; dermatoses with acute inflammatory components; acute purulent meningitis or other central nervous system inflammatory disorders; thermal injury; hemodialysis; leukapheresis; ulcerative colitis; Crohn's disease; necrotizing enterocolitis; granulocyte transfusion associated syndromes; and cytokine-induced toxicity. Examples of autoimmune diseases include, but are not limited to psoriasis, Type I diabetes, Reynaud's syndrome, autoimmune thyroiditis, EAE, multiple sclerosis, rheumatoid arthritis and lupus erythematosus.

[000172] As an example, Tables 4 through 7 lists a number of genes from which mRNA is transcribed, that may be modulated by siRNA; table 4 (CD markers), table 5 (adhesion molecules) table 6 (chemokines and chemokine receptors), and table 7 (interleukins and their receptors). Also included are the genes encoding the immunoglobulin E (IgE) and the IgE-receptor (FcεRIα) as well as the genes for the other immunoglobulins, IgG₍₁₋₄₎, IgA₁, IgA₂,

IgM, IgE, and IgD encoding free and membrane bound immunoglobulins and the genes encoding their corresponding receptors.

Table 4

CD markers				
CD1a-d	CD30	CD61	CD91	CD121
CD2	CD31	CD62E	CDw92	CD122
CD3	CD32	CD62L	CD93	CDw123
CD4	CD33	CD62P	CD94	CD124
CD5	CD34	CD63	CD95	CDw125
CD6	CD35	CD64	CD96	CD126
CD7	CD36	CD65	CD97	CD127
CD8	CD37	CD66a-e	CD98	CDw128
CD9	CD38	CD67	CD99	CD129
CD10	CD39	CD68	CD100	CD130
CD11a	CD40	CD69	CD101	CDw131
CD11b	CD41	CD70	CD102	CD132
CD11c	CD42a-d	CD71	CD103	CD133
CDw12	CD43	CD72	CD104	CD134
CD13	CD44	CD73	CD105	
CD14	CD45	CD74	CD106	
CD15	CD46	CDw75	CD107a,b	
CD16	CD47	CDw76	CDw08	
CDw17	CD48	CD77	CD109	
CD18	CD49a-f	CDw78	CD110	
CD19	CD50	CD79a,b	CD111	
CD20	CD51	CD80	CD112	
CD21	CD52	CD81	CD113	
CD22	CD53	CD82	CD114	
CD23	CD54	CD83	CD115	
CD24	CD55	CDw84	CD116	
CD25	CD56	CD85	CD117	
CD26	CD57	CD86	CD118	
CD27	CD58	CD87	CD119	
CD28	CD59	CD88	CD120a,b	
CD29	CDw60	CD89		
CD30		CD90		

Table 5

Adhesion molecules				
L-selectin	TCR γ/δ	BB-1	Integrin $\alpha 7$	Integrin $\alpha 6$
P- selectin	CD28	N-cadherin	Integrin $\alpha 8$	Integrin $\beta 5$
E-selectin	LFA-3	E-cadherin P-	Integrin αV	Integrin αV
HNK-1	PECAM-1	cadherin	Integrin $\beta 2$	Integrin $\beta 6$
Sialyl-Lewis X	VCAM-1	Integrin $\beta 1$	Integrin αL	Integrin αV
CD15	ICAM-2	Integrin $\alpha 1$	Integrin αM	Integrin $\beta 7$
LFA-2	ICAM-3	Integrin $\alpha 2$	Integrin αX	
CD22	Leukosialin	Integrin $\alpha 3$	Integrin $\beta 3$	Integrin αIEL
ICAM-1	HCAM	Integrin $\alpha 4$	Integrin αV	Integrin $\alpha 4$
N-CAM	CD45RO	Integrin $\alpha 5$	Integrin αIib	Integrin $\beta 8$
Ng-CAM	CD5	Integrin $\alpha 6$	Integrin $\beta 4$	Integrin αV
TCR α/β	HPCA-2			

Table 6

Chemokines and Chemokine receptors				
C-X-C chemokines	C-C chemokines		C chemokines	Chemokine Receptors
IL-8	MCAF/MCP-1	ABCD-1	<i>Lymphotactin</i>	CCR1
NAP-2	MIP-1 α, β	LMC		CCR2
GRO/MGSA	RANTES	AMAC-1		CCR3
γ IP-10	I-309	NCC-4		CCR4
ENA-78	CCF18	LKN-1		CCR5
SDF-1	SLC	STCP-1		CCR6
I-TAC	TARC	TECK		CCR7
LIX	PARC	EST		CCR8
SCYB9	LARC	MDC		CXCR1
B cell-attracting chemokine 1	EBI 1	Eotaxin		CXCR2
	HCC-1			CXCR3
	HCC-4			CXCR4
				CXCR5
				CX ₃ CR

Table 7

Interleukins and their receptors				
G-CSF	IL-2 R α	IL-8	IL-16	TGF- β 1
G-CSF R	IL-2 R β	IL-9	IL-17	TGF- β 1,2
GM-CSF	IL-2 R γ	IL-9 R	IL-18	TGF- β 2
IFN- γ	IL-3	IL-10	PDGF	TGF- β 3
	IL-3 R α	IL-10 R	PDGF A Chain	TGF- β 5
IGF-I	IL-4	IL-11	PDGF-AA	LAP TGF- β 1
IGF-I R	IL-4 R	IL-11 R	PDGF-AB	Latent TGF- β 1
	IL-5	IL-12	PDGF B Chain	TGF- β b.p.1
IGF-II	IL-5 R α	IL-12 p40	PDGF-BB	TGF- β RII
IL-1 α	IL-6	IL-12 p70	PDGF R α	TGF- β RIII
IL-1 β	IL-6 R	IL-13	PDGF R β	
IL-1 RI	IL-7	IL-13 R α	TGF- α	
IL-1 RII	IL-7 R	IL-15	TGF- β	
IL-1 α				
IL-2				

[000173] It should be appreciated that in the above tables 4 through 7, an indicated gene means the gene and all currently known variants thereof, including the different mRNA transcripts to which the gene and its variants can give rise, and any further gene variants which may be elucidated. In general, however, such variants will have significant homology (sequence identity) to a sequence of a table above, e.g. a variant will have at least about 70 percent homology (sequence identity) to a sequence of the above tables 1-5, more typically at least about 75, 80, 85, 90, 95, 97, 98 or 99 homology (sequence identity) to a sequence of the above tables 7 - 10. Homology of a variant can be determined by any of a number of standard techniques such as a BLAST program.

[000174] Sequences for the genes listed in Tables 7 - 10 can be found in GenBank (<http://www.ncbi.nlm.nih.gov/>). The gene sequences may be genomic, cDNA or mRNA sequences. Preferred sequences are mammalian genes comprising the complete coding region and 5' untranslated sequences. Particularly preferred are human cDNA sequences.

[000175] The methods of the invention can be used to screen for siRNA polynucleotides that inhibit the functional expression of one or more genes that modulate immune related molecule expression. For example, the CD-18 family of molecules is important in cellular adhesion. Through the process of adhesion, lymphocytes are capable of continually monitoring an animal for the presence of foreign antigens. Although these processes are normally desirable, they are also the cause of organ transplant rejection, tissue graft rejection

and many autoimmune diseases. Hence, siRNA's capable of attenuating or inhibiting cellular adhesion would be highly desirable in recipients of organ transplants (for example, kidney transplants), tissue grafts, or for autoimmune patients.

[000176] In another preferred embodiment, siRNA oligonucleotides inhibit the expression of MHC molecules involved in organ transplantation or tissue grafting. For example, Class I and Class II molecules of the donor. siRNA inhibit the expression of these molecules thereby ameliorating an allograft reaction. Immune cells may be treated prior to the organ or tissue transplantation, administered at time of transplantation and/or any time thereafter, at times as may be determined by an attending physician. siRNAs can be administered with or without immunosuppressive drug therapy.

[000177] In another preferred embodiment, siRNA's are used to treat individuals who are hyper-responsive to an antigen such as an allergic individual. siRNA's are designed to target V region genes known to produce IgE molecules specific for the allergen. IgE antibody specificity can be determined by routine immuno diagnostic techniques such as ELISA's, RIA's, PCR, Western Blots etc. From the amino acid sequence of the IgE molecules, the nucleic acid sequence can be deduced, using any of the database techniques described infra. siRNA's are designed to bind to V region genes or any other part of a gene that makes encodes for the desired antibody, including rearranged and non-rearranged immunoglobulin nucleic acid sequences.

[000178] In another preferred embodiment, siRNA's are designed to target suppressor molecules that suppress the expression of gene that is not suppressed in a normal individual. For example, suppressor molecules which inhibit cell-cycle dependent genes, inhibition of p53 mRNA, inhibition of mRNA transcribed by genes coding for cell surface molecules (see tables 7-10), inhibition of caspases involved in apoptosis and the like.

[000179] Apoptosis is important clinically for several reasons. In the field of oncology, many of the clinically useful drugs kill tumor cells by inducing apoptosis. For example, cancer chemotherapeutic agents such as cisplatin, etoposide and taxol all induce apoptosis in target cells. In addition, a variety of pathological disease states can result from the failure of cells to undergo proper regulated apoptosis. For example, the failure to undergo apoptosis can lead to the pathological accumulation of self-reactive lymphocytes such as that occurring in many autoimmune diseases, and can also lead to the accumulation of virally infected cells and to the accumulation of hyperproliferative cells such as neoplastic or tumor cells. siRNA's which target mRNA's from which proteins are translated and are capable of specifically

inducing apoptosis would therefore be of therapeutic value in the treatment of these pathological disease states.

[000180] In contrast, the inhibition of apoptosis is also of clinical importance. For example, cells are thought to die by apoptosis in the brain and heart following stroke and myocardial infarction, respectively. Moreover, the inappropriate activation of apoptosis can also contribute to a variety of other pathological disease states including, for example, acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases and ischemic injuries. As apoptotic inducers are of benefit in the previously mentioned disease states, specific inhibitors of apoptosis would similarly be of therapeutic value in the treatment of these latter pathological disease states.

[000181] In a preferred embodiment, siRNA's target genes that prevent the normal expression or, if desired, over expression of genes that are of therapeutic interest as described above. As used herein, the term "overexpressing" when used in reference to the level of a gene expression is intended to mean an increased accumulation of the gene product in the overexpressing cells compared to their levels in counterpart normal cells. Overexpression can be achieved by natural biological phenomenon as well as by specific modifications as is the case with genetically engineered cells. Overexpression also includes the achievement of an increase in cell survival polypeptide by either endogenous or exogenous mechanisms. Overexpression by natural phenomenon can result by, for example, a mutation which increases expression, processing, transport, translation or stability of the RNA as well as mutations which result in increased stability or decreased degradation of the polypeptide. Such examples of increased expression levels are also examples of endogenous mechanisms of overexpression. A specific example of a natural biologic phenomenon which results in overexpression by exogenous mechanisms is the adjacent integration of a retrovirus or transposon. Overexpression by specific modification can be achieved by, for example, the use of siRNA oligonucleotides described herein.

[000182] An siRNA polynucleotide may be constructed in a number of different ways provided that it is capable of interfering with the expression of a target protein. The siRNA polynucleotide generally will be substantially identical (although in a complementary orientation) to the target molecule sequence. The minimal identity will typically be greater than about 80%, greater than about 90%, greater than about 95% or about 100% identical.

Delivery of siRNA

[000183] Preferred invention practice involves administering at least one of the foregoing siRNA polynucleotides with a suitable nucleic acid delivery system. In one embodiment, that system includes a non-viral vector operably linked to the polynucleotide. Examples of such non-viral vectors include the polynucleoside alone or in combination with a suitable protein, polysaccharide or lipid formulation.

[000184] Additionally suitable nucleic acid delivery systems include viral vector, typically sequence from at least one of an adenovirus, adenovirus-associated virus (AAV), helper-dependent adenovirus, retrovirus, or hemagglutinating virus of Japan-liposome (HVJ) complex. Preferably, the viral vector comprises a strong eukaryotic promoter operably linked to the polynucleotide e.g., a cytomegalovirus (CMV) promoter.

[000185] As an illustrative example which is not meant to limit or construe the invention in any way, a vector was constructed using parent plasmid p21NewHP and synthetic oligonucleotides encoding Hammerhead ribozyme (Rz) and RNAi targeting for GFP reporter gene. The respective nucleotide sequences are identified by SEQ ID NO's: 1 through 4. This provides a DNA cassette for the cloning of small hairpin sequences which permit their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and permit the use of regulated and tissue specific promoters for gene expression.

[000186] The cassette comprises hammerhead and hairpin ribozymes flanking the cloning sites for hairpin RNA of variable sequence, depending on the target of RNA interference. The hairpin RNA, is processed out through the autocatalytic activities of the ribozymes which cleave at the sites indicated by the arrows. These leave 6 extra nucleotides at the 5' end of the hairpin and 9 extra at the 3' end. The cloning site for insertion of the hairpin RNA can deviate from the prototype shown here, i.e. the sequence can be varied depending on the desired target nucleic acid molecule sequence, as long as internal base-pairing is preserved in the flanking hammerhead ribozyme (Rz) and hairpin ribozyme. Examples of desired targets are described above.

[000187] Figure 2 shows an illustrative example of a vector encoding an siRNA of the invention. The cassette was cloned in an AAV (Adeno-associated virus) vector in conjunction with the chicken beta actin promoter and the CMV immediate early enhancer to create pT-RRR. Digestion with *Hind*III and *Spe*I can be used to insert the genes for hairpin RNAs for delivery by recombinant AAV.

[000188] Additionally preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-

based viruses. One preferred HIV-based viral vector comprises at least two vectors wherein the gag and pol genes are from an HIV genome and the env gene is from another virus. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as a herpes simplex I virus (HSV) vector [Geller, A.I. et al., *J. Neurochem*, 64: 487 (1995); Lim, F., et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A.I. et al., *Proc Natl. Acad. Sci.*: U.S.A.:90 7603 (1993); Geller, A.I., et al., *Proc Natl. Acad. Sci USA*: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet.* 3: 219 (1993); Yang, et al., *J. Virol.* 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.* 8:148 (1994)].

[000189] Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors may be an indication for some invention embodiments. The adenovirus vector results in a shorter term expression (e.g., less than about a month) than adeno-associated virus; in some embodiments, may exhibit much longer expression. The particular vector chosen will depend upon the target cell and the condition being treated. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a tat gene and tar element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as, pUC19, pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). Promoters are discussed *infra*. The plasmid vector may also include a selectable marker such as the β -lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

[000190] If desired, the polynucleotides of the invention may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and

Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R.A., Bethesda Res. Lab. Focus, 11(2):25 (1989).

[000191] Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584 (1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

[000192] Another preferred siRNA delivery method is to use single stranded DNA producing vectors which can produce the siRNA's intracellularly. See for example, Chen et al, *BioTechniques*, 34: 167-171 (2003), which is incorporated herein, by reference, in its entirety.

[000193] The effective dose of the nucleic acid will be a function of the particular expressed protein, the particular cardiac arrhythmia to be targeted, the patient and his or her clinical condition, weight, age, sex, etc.

[000194] One preferred delivery system is a recombinant viral vector that incorporates one or more of the polynucleotides therein, preferably about one polynucleotide. Preferably, the viral vector used in the invention methods has a pfu (plaque forming units) of from about 10^8 to about 5×10^{10} pfu. In embodiments in which the polynucleotide is to be administered with a non-viral vector, use of between from about 0.1 nanograms to about 4000 micrograms will often be useful e.g., about 1 nanogram to about 100 micrograms.

Promoters

[000195] In a preferred embodiment, the expression cassette is expressed and processed by RNA polymerase II. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

[000196] The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct

location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

[000197] The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 b.p. of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

[000198] At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

[000199] Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 b.p. upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 b.p. apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

[000200] The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

[000201] In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β -actin, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or

mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

[000202] Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic.

[000203] The ecdysone system (Invitrogen, Carlsbad, Calif.) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of *Drosophila*, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constitutively expressed from one vector, whereas the ecdysone-responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would then allow for the production of the gene transfer vector without expression of a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristerone A.

[000204] Another inducible system that would be useful is the Tet-OffTM or Tet-OnTM system (Clontech, Palo Alto, Calif.). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-OnTM system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-OffTM system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli*. The tetracycline operator sequence to which the tetracycline repressor binds, and

the tetracycline repressor protein. The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-OffTM system, of the VP16 domain from the herpes simplex virus and the wild-type tetracycline repressor. Thus in the absence of doxycycline, transcription is constitutively on. In the Tet-OnTM system, the tetracycline repressor is not wild type and in the presence of doxycycline activates transcription. For gene therapy vector production, the Tet-OffTM system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constitutively on.

[000205] In some circumstances, it may be desirable to regulate expression of a transgene in a gene therapy vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter is often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of a transgene in hematopoietic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic Virus, HSV-TK, and avian sarcoma virus.

[000206] In a preferred embodiment, tissue specific promoters are used to effect transcription in specific tissues or cells so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate. Similarly, the following promoters may be used to target gene expression in other tissues (Table 8).

[000207]

TABLE 8

Tissue specific promoters

Tissue	Promoter
Pancreas	insulin elastin amylase pdr-1 pdx-1 glucokinase
Liver	albumin PEPCK HBV enhancer alpha fetoprotein apolipoprotein C alpha-1 antitrypsin vitellogenin, NF-AB Transthyretin
Skeletal muscle	myosin H chain muscle creatine kinase dystrophin calpain p94 skeletal alpha-actin fast troponin 1
Skin	keratin K6 keratin K1
Lung	CFTR human cytokeratin 18 (K18) pulmonary surfactant proteins A, B and C CC-10 P1
Smooth muscle	sm22 alpha SM-alpha-actin
Endothelium	endothelin-1 E-selectin von Willebrand factor TIE (Korhonen et al., 1995) KDR/flk-1
Melanocytes	tyrosinase
Adipose tissue	lipoprotein lipase adipsin acetyl-CoA carboxylase glycerophosphate dehydrogenase adipocyte P2
Blood	β -globin

[000208] In certain indications, it may be desirable to activate transcription at specific times after administration of the gene therapy vector. This may be done with such promoters as those that are hormone or cytokine regulatable. For example in gene therapy applications where the indication is a gonadal tissue where specific steroids are produced or routed to, use

of androgen or estrogen regulated promoters may be advantageous. Such promoters that are hormone regulatable include MMTV, MT-1, ecdysone and RuBisco. Other hormone regulated promoters such as those responsive to thyroid, pituitary and adrenal hormones are expected to be useful in the present invention. Cytokine and inflammatory protein responsive promoters that could be used include K and T Kininogen, c-fos, TNF-alpha, C-reactive protein, haptoglobin, serum amyloid A2, C/EB.P. alpha, IL-1, IL-6, Complement C3, IL-8, alpha-1 acid glycoprotein, alpha-1 antitrypsin, lipoprotein lipase, angiotensinogen, fibrinogen, c-jun (inducible by phorbol esters, TNF-alpha, UV radiation, retinoic acid, and hydrogen peroxide), collagenase (induced by phorbol esters and retinoic acid), metallothionein (heavy metal and glucocorticoid inducible), Stromelysin (inducible by phorbol ester, interleukin-1 and EGF), alpha-2 macroglobulin and alpha-1 antichymotrypsin.

[000209] It is envisioned that cell cycle regulatable promoters may be useful in the present invention. For example, in a bi-cistronic gene therapy vector, use of a strong CMV promoter to drive expression of a first gene such as p16 that arrests cells in the G1 phase could be followed by expression of a second gene such as p53 under the control of a promoter that is active in the G1 phase of the cell cycle, thus providing a "second hit" that would push the cell into apoptosis. Other promoters such as those of various cyclins, PCNA, galectin-3, E2F1, p53 and BRCA1 could be used.

[000210] Tumor specific promoters such as osteocalcin, hypoxia-responsive element (HRE), MAGE-4, CEA, alpha-fetoprotein, GRP78/BiP and tyrosinase may also be used to regulate gene expression in tumor cells. Other promoters that could be used according to the present invention include Lac-regulatable, chemotherapy inducible (e.g. MDR), and heat (hyperthermia) inducible promoters, radiation-inducible (e.g., EGR (Joki et al., 1995)), Alpha-inhibin, RNA pol III tRNA met and other amino acid promoters, U1 snRNA (Bartlett et al., 1996), MC-1, PGK, β -actin and α -globin. Many other promoters that may be useful are listed in Walther and Stein (1996).

[000211] It is envisioned that any of the above promoters alone or in combination with another may be useful according to the present invention depending on the action desired. In addition, this list of promoters is should not be construed to be exhaustive or limiting, those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

Enhancers

[000212] Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

[000213] Below is a list of promoters additional to the tissue specific promoters listed above, cellular promoters/enhancers and inducible promoters/enhancers that could be; used in combination with the nucleic acid encoding a gene of interest in an expression construct. Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 9
ENHANCER

Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β -Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II HLA-DR α
β -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase I
Metallothionein
Collagenase
Albumin Gene
α -Fetoprotein
τ -Globin
β -Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α 1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen

Glucose-Regulated Proteins (GRP94 and GRP78)
 Rat Growth Hormone
 Human Serum Amyloid A (SAA)
 Troponin I (TN I)
 Platelet-Derived Growth Factor
 Duchenne Muscular Dystrophy
 SV40
 Polyoma
 Retroviruses
 Papilloma Virus
 Hepatitis B Virus
 Human Immunodeficiency Virus
 Cytomegalovirus
 Gibbon Ape Leukemia Virus

TABLE 10

<u>Element</u>	<u>Inducer</u>
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β -Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	EIA
c-jun	Phorbol Ester (TPA), H ₂ O ₂
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	EIA, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone Gene	α -Thyroid Hormone
Insulin E Box	Glucose

[000214] In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells. The first viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up

to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

Polyadenylation Signals

[000215] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

IRES

[000216] In certain embodiments of the invention, the use of internal ribosome entry site (IRES) elements is contemplated to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites. IRES elements from two members of the picornavirus family (poliovirus and encephalomyocarditis) have been described, as well an IRES from a mammalian message. IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

[000217] Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

Assessing Gene Silencing

[000218] Transfer of an exogenous nucleic acid into a host cell or organism by a vector can be assessed by directly detecting the presence of the nucleic acid in the cell or organism. Such detection can be achieved by several methods well known in the art. For example, the presence of the exogenous nucleic acid can be detected by Southern blot or by a polymerase

chain reaction (PCR) technique using primers that specifically amplify nucleotide sequences associated with the nucleic acid. Expression of the exogenous nucleic acids can also be measured using conventional methods. For instance, mRNA produced from an exogenous nucleic acid can be detected and quantified using a Northern blot and reverse transcription PCR (RT-PCR).

[000219] Expression of an RNA from the exogenous nucleic acid can also be detected by measuring an enzymatic activity or a reporter protein activity. For example, siRNA activity can be measured indirectly as a decrease in target nucleic acid expression as an indication that the exogenous nucleic acid is producing the effector RNA.

Applications to Plants

[000220] In another preferred embodiment, the siRNA's are designed to target mRNA in a plant. The targeted nucleic acid express an enzyme, a plant structural protein, a molecule involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

[000221] The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in Index of plant Diseases in the United States (U.S. Dept. of Agriculture Handbook No. 165, 1960); Distribution of Plant-Parasitic Nematode Species in North America (Society of Nematologists, 1985); and Fungi on Plants and Plant Products in the United States (American Phytopathological Society, 1989). Inhibition of target gene activity could be used to delay or prevent entry of an infectious disease organism into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle.

[000222] Introduction of the siRNA's into plants can be achieved in many ways. In the past decade, a number of techniques have been developed to transfer genes into plants (Potrykus, I., *Annual Rev. Plant Physiol. Plant Mol. Biol.* 42:205-225 (1991)). For example, chromosomally integrated transgenes have been expressed by a variety of promoters offering developmental control of gene expression. (Walden and Schell, *Eur. J. Biochem.* 192:563-576 (1990)). The most highly expressed genes in plants are encoded in plant RNA viral

genomes. Many RNA viruses have gene expression levels or host ranges that make them useful for development as commercial vectors. (Ahlquist, P., and Pacha, R. F., *Physiol. Plant.* 79:163-167 (1990), Joshi, R. L., and Joshi, V., *FEBS Lett.* 281:1-8 (1991), Turpen, T. H., and Dawson, W. O., Amplification, movement and expression of genes in plants by viral-based vectors, *Transgenic plants: fundamentals and applications* (A. Hiatt, ed.), Marcel Dekker, Inc., New York, pp. 195-217. (1992)). For example, tobacco (*Nicotiana tabacum*) accumulates approximately 10 mg of tobacco mosaic tobamovirus (TMV) per gram of fresh-weight tissue 7-14 days after inoculation. TMV coat protein synthesis can represent 70% of the total cellular protein synthesis and can constitute 10% of the total leaf dry weight. A single specific RNA transcript can accumulate to 10% of the total leaf mRNA. This transcript level is over two orders of magnitude higher than the transcription level observed for chromosomally integrated genes using conventional plant genetic engineering technology. Most plant viruses contain genomes of plus sense RNA (messenger RNA polarity) (Zaitlin and Hull, *Ann. Rev. Plant Physiol.* 38:291-315 (1987)). Plus sense plant viruses are a very versatile class of viruses to develop as gene expression vectors since there are a large number of strains from some 22 plus sense viral groups which are compatible with a wide number of host plant species. (Martelli, G. P., *Plant Disease* 76:436 (1992)). In addition, an evolutionarily related RNA-dependent RNA polymerase is encoded by each of these strains. This enzyme is responsible for genome replication and mRNA synthesis resulting in some of the highest levels of gene expression known in plants.

[000223] In order to develop a plant virus as a gene vector, one must be able to manipulate molecular clones of viral genomes and retain the ability to generate infectious recombinants. The techniques required to genetically engineer RNA viruses have progressed rapidly. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is used to make all of the constructions. The genome of many plus sense RNA viruses can be manipulated as plasmid DNA copies and then transcribed in vitro to produce infectious RNA molecules (reviewed in Turpen and Dawson, *Transgenic Plants, Fundamentals and Applications*, Marcel Dekker, New York, pp. 195-217 (1992)).

[000224] The interaction of plants with viruses presents unique opportunities for the production of complex molecules as typified by the TMV/tobacco system (Dawson, W. O., *Virology* 186:359-367 (1992)). Extremely high levels of viral nucleic acids and/or proteins accumulate in infected cells in a brief period of time. The virus catalyzes rapid cell-to-cell movement of its genome throughout the plant, with no significant tissue tropism. The infection is maintained throughout the life of the plant. The plants are not significantly

adversely affected by the viral infection since the virus causes little or no general cytotoxicity or specific suppression of host gene expression.

[000225] In another preferred embodiment, ribozyme-siRNA cassettes are transduced into plants and/or plant cells using vectors such as, for example, a tumor inducing (Ti) plasmid or portion thereof found in the bacterium *Agrobacterium*. A portion of the Ti plasmid is transferred from the bacterium to plant cells when *Agrobacterium* infects plants and produces a crown gall tumor. This transferred DNA is hereinafter referred to as "transfer DNA" (T-DNA). The transfer DNA integrates into the plant chromosomal DNA and can be shown to express the genes carried in the transferred DNA under appropriate conditions. Another example, employs cauliflower mosaic virus (CaMV) DNA as a vector for introduction of desired siRNA oligonucleotide sequences into plant cells. CaMV is a member of the caulimovirus group and contains a double-stranded DNA genome.

[000226] Another technique in which ribozyme-siRNA cassettes can be transduced into plant cells is by called electroporation.

[000227] In another preferred embodiment, ribozyme-siRNA cassettes can be introduced into plants using viruses with a DNA genome. One group of plant viruses has been identified which contains a DNA, rather than RNA genome. This group comprises the geminiviruses. Geminiviruses are plant viruses characterized by dumbbell-shaped twinned icosahedral particles (seen by electron micrograph). Some geminiviruses comprise two distinct circular single-stranded (ss) DNA genomes. Examples of such two genome or binary geminiviruses include tomato golden mosaic virus (TGMV) which has an "A" DNA and a "B" DNA, and Cassava latent virus (CLV) which has a "1" DNA and a "2" DNA. Other geminiviruses such as maize streak virus (MSV) are believed to have a single circular ssDNA genome. Typically, two genome (binary) geminiviruses are transmitted by white flies, while single genome geminiviruses are transmitted by leaf hoppers. As a group, geminiviruses infect both monocotyledonous and dicotyledonous plants and thus exhibit a broad host range.

[000228] All geminivirus particles carry circular ssDNA. In infected plant cells, geminivirus DNA sequences have been detected as both ss and double-stranded (ds) DNA, in predominately circular form. In infected plants, such sequences exist in the plant cell nuclei, apparently as episomes, at several hundred copies per nuclei. Thus, unlike the transfer DNA (T-DNA) derived from the Ti plasmids of *Agrobacterium*, these geminivirus DNA sequences are not integrated into plant chromosomal DNA and generate multiple copies (e.g. more than 5) per infected cell. In infected plants, geminivirus particles released by an infected cell can infect other cells throughout the plant. In the two genome geminivirus systems such as

TGMV, infectivity, replication and movement throughout the whole plant has thus far been shown to require the presence of both the A and B components. These vectors are described in U.S. Patent No.: 6,147,278 which is incorporated herein by reference.

[000229] The practice of the present invention can suitably employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al. eds., 1987 and updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al. eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology* (R. Wu et al. eds., Academic Press 1989); *PCR: A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al. eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al. eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al. eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); *Techniques in Immunocytochemistry*, (G. Bul & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); *Handbook of Experimental Immunology*, (D. Weir & C. Blackwell, eds.); *Cellular and Molecular Immunology* (A. Abbas et al., W. B. Saunders Co. 1991, 1994); *Current Protocols in Immunology* (J. Coligan et al. eds. 1991); the series *Annual Review of Immunology*; the series *Advances in Immunology*; *Oligonucleotide Synthesis* (M. Gait ed., 1984); and *Animal Cell Culture* (R. Freshney ed., IRL Press 1987).

Sequence Alignments

[000230] Methods of alignment of sequences for comparison and to identify previously unidentified sequences, are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci USA* 85: 2444 (1988), by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by

Intelligenetics, Mountain View, Calif., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., USA), or by inspection. In particular, methods for aligning sequences using the CLUSTAL program are well described by Higgins and Sharp in *Gene*, 73: 237-244 (1988) and in *CABIOS* 5: 151-153 (1989)).

Target Validation

[000231] The nucleic acid molecules of the present invention can inhibit gene expression in a highly specific manner by binding to and causing the cleavage of the mRNA corresponding to the gene of interest, and thereby prevent production of the gene product (Christoffersen, *Nature Biotech*, 1997, 2, 483-484). Appropriate delivery vehicles can be combined with these nucleic acid molecules (including polymers, cationic lipids, liposomes and the like) and delivered to appropriate cell culture or in vivo animal disease models as described above. By monitoring inhibition of gene expression and correlation with phenotypic results, the relative importance of the particular gene sequence to disease pathology can be established. The process can be both fast and highly selective, and allow for the process to be used at any point in the development of the organism. The novel chemical composition of these nucleic acid molecules can allow for added stability and therefore increased efficacy.

Other Embodiments

[000232] The foregoing paragraphs have described a preferred embodiment in which separate sense and antisense RNA molecules are synthesized and RNAi is produced through intermolecular hybridization of complementary sequences. As those skilled in the art will readily appreciate, RNAi can also be produced through intramolecular hybridization of complementary regions within a single RNA molecule. An expression unit for synthesis of such a molecule comprises the following elements, positioned from left to right: 1. A DNA region comprising a viral enhancer; 2. A DNA region comprising an immediate early or early viral promoter oriented in a 5' to 3' direction so that a DNA segment inserted into the region of part 4 is transcribed; 3. A DNA region into which a DNA segment can be inserted. Preferably this region contains at least one restriction enzyme site; 4. A DNA region comprising a transcriptional terminator arranged in a 5' to 3' orientation so that a transcript synthesized in a left to right direction from the promoter of part 2 is terminated.

Kits

[000233] In yet another aspect, the invention provides kits for targeting nucleic acid sequences of infectious disease organism, cancer, autoimmune diseases and the like. For example, the kits can be used to target any desired nucleic sequence, such as an HPV sequence. The kits of the invention have many applications. For example, the kits can be used to target and kill cells infected with a virus, or if the cells are at different stages of a tumor. In another example, the kits can be used to treat patients with a particular disease.

[000234] In one embodiment, a kit comprises: (a) a Ribozyme-siRNA cassette that targets a desired nucleic acid sequence, and (b) instructions to administer to cells or an individual a therapeutically effective amount of cassettes. In some embodiments, the kit may comprise pharmaceutically acceptable salts or solutions for administering the ribozyme-siRNA cassette s.

[000235] Optionally, the kit can further comprise instructions for suitable operational parameters in the form of a label or a separate insert. For example, the kit may have standard instructions informing a physician or laboratory technician to prepare a dose of ribozyme-siRNA cassettes. In another example, the kit may have instructions for treating a plant infected with virus, fungus and the like.

[000236] Optionally, the kit may further comprise a standard or control information so that a patient sample can be compared with the control information standard to determine if the test amount of a ribozyme-siRNA cassette is a therapeutic amount consistent with for example, a shrinking of a tumor or decrease in viral load in a patient.

[000237] All documents mentioned herein are incorporated herein by reference. All publications and patent documents cited in this application are incorporated by reference for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

EXAMPLES

[000238] The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention. The following non-limiting examples are illustrative of the invention.

Materials and Methods

[000239] *Transfections and Flow Cytometry:* Transfections were done using HEK 293 cells. 24 hours prior to transfections approximately 6×10^5 cells were plated in a 6-well plate. During the transfection all plates were transfected with 0.5 μg regulated eGFP plasmid and 0.2 μg of plasmid expressing the tet trans-activator (tta). The experimental groups were as follows: 1.5 μg RRRsiRNA, 1.0 μg RRRsiRNA, and 1.5 μg of plasmid with the same siRNA sequence expressed under a U6 promoter, as well as a group with no siRNA. All these groups received the same amount of GFP and tta plasmids. The total amount of transfected DNA was normalized using a plasmid with no siRNA or transgene and diluted with 100 μl of OptiMem™. 20 μl of Qiagen Polyfect™ reagent was added to the DNA mixture and allowed to incubate at room temperature for 15 minutes. After the incubation 600 μl OptiMem™ was added and the entire mixture was added to the cells. 24 hours after the transfection doxycycline was added to the media with a final concentration of 10 $\mu\text{g}/\text{ml}$. All the transfections were done in triplicate. At 24, 48, or 72 hours following the GFP gene induction the cells were harvested for flow-cytometry analysis of GFP positive cells. The cells were trypsinized and washed 3 times in phosphate buffered saline (PBS) and resuspended in 1 ml PBS and immediately analyzed using flow-cytometry. (See, Figure 3).

[000240] *Stereotaxic rAAV injection:* Adult female Sprague–Dawley rats were used, and all surgical procedures were performed using aseptic techniques and an isoflurane gas anesthesia apparatus. After the rats were anesthetized, they were injected in the right substantia nigra pars compacta using a stereotaxic frame and isoflurane anesthesia during the injection procedure. The brain coordinates for the injections were, anterior–posterior (AP) –5.3 mm, lateral (Lat) –1.0 mm, dorsoventral (DV) –7.2 mm from dura. Injections were performed with a 5- μl Hamilton syringe fitted with a glass micropipette with an opening of approximately 60–80 μm . Two microliters were injected per brain structure at a rate of 0.5 $\mu\text{l}/\text{min}$. The rate of injection was precisely controlled by an infusion pump that pushes a piston, which in turn depresses the plunger on the Hamilton syringe. The needle was left in place for 5 min prior to withdrawal from the brain. (See, Figure 4).

[000241] *Histology:* Four weeks after virus injections, animals were deeply anesthetized and perfused through the ascending aorta. Brains were perfused with 50 ml of isotonic saline, followed by 250 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed and postfixed for 2 h in the same solution. The brains were then transferred to 20% sucrose in 0.1 M PB for cryoprotection. Forty-micrometer-thick

coronal sections were cut in series of 5 on a freezing stage sliding microtome, and processed for immunohistochemistry. (See Figure 4).

Example 1: Expression of functional intracellular siRNA

[000242] RNA interference (RNAi) has become a powerful tool for blocking gene expression in mammals and mammalian cells. This approach requires the delivery of small interfering RNA (siRNA) either as RNA itself or as DNA, using an expression plasmid or virus and the coding sequence for small hairpin RNAs that are processed to siRNAs. Current expression systems for the production of siRNA *in vivo* rely on RNA polymerase III promoters. These are difficult to regulate and leave most of the RNA in the nucleus of the cell where it is inactive for RNA interference. This invention provides a DNA cassette for the cloning of small hairpin sequences which permit their expression and processing using RNA polymerase II. This system enables efficient transport of the pre-siRNAs to the cytoplasm where they are active and permit the use of regulated and tissue specific promoters for gene expression.

[000243] The cassette comprises hammerhead and hairpin ribozymes flanking the cloning sites for hairpin RNA of variable sequence, depending on the target of RNA interference. The hairpin RNA, here shown in red (GFP RNAi), is processed out through the autocatalytic activities of the ribozymes which cleave at the sites indicated by the arrows. These leave 6 extra nucleotides at the 5' end of the hairpin and 9 extra at the 3' end. The cloning site for insertion of the hairpin RNA can deviate from the prototype shown here as long as internal base-pairing is preserved in the flanking hammerhead ribozyme (Rz) and hairpin ribozyme.

[000244] Figure 2 shows an illustrative example of a vector encoding an siRNA of the invention. The cassette was cloned in an AAV (Adeno-associated virus) vector in conjunction with the chicken beta actin promoter and the CMV immediate early enhancer to create pT-RRR. Digestion with *HindIII* and *SpeI* can be used to insert the genes for hairpin RNAs for delivery by recombinant AAV.

[000245] Transfection of this plasmid plus a plasmid expressing GFP in 293 cells led to a 3-fold reduction in the number of cells expressing GFP at 48 hours post transfection and a 60% reduction at 72 hours post transfection based on FACS analysis, suggesting that this plasmid can lead to the production of functional siRNA in cultured cells. See Figure 3.

[000246] Figure 4 shows the results obtained when the plasmid is transfected into cells. A small hairpin RNA (shRNA) specific for the mRNA encoding tyrosine hydroxylase (TH) was inserted into the pT-RRR vector in place of the GFP specific hairpin. Tyrosine

hydroxylase is needed for the production of the neurotransmitter dopamine. When this plasmid was packaged in AAV and injected into the striatum of rats, a 50% reduction in accumulation of this enzyme was observed 4 weeks after the viral injection. This observation indicates that the cassette can be used for the production of functional siRNA *in vivo*.

[000247] This cassette expression system can be used in conjunction with a variety of promoters that are either tissue specific, e.g. myosin heavy chain promoter for skeletal muscle, rhodopsin promoter for the eye, or are regulated by small molecules, e.g. tetracycline regulated promoters, ecdysone regulated promoters or rapamycin regulated promoters. This permits RNA interference mediated reduction in gene expression mediated by tissue specific or regulated expression of small hairpin RNAs. Delivery would likely be using viral vectors such as AAV or lentivirus, but could also be by transfected or electroporated plasmid DNA.

[000248] Applications of this invention would include gene therapy for dominantly inherited genetic diseases such as Parkinson Disease, Huntington Disease, Autosomal Dominant Retinitis Pigmentosa, Familial Hypertrophic Cardiomyopathy etc. The cassette could also be used in strategies to use RNA interference to treat viral infections, such as herpes simplex viruses or cytomegalovirus, which become latent in the human host and may re-activate at any time. In addition, this technology could have widespread research application for the development of animal models of human disease and for studying gene function in animals and in tissue culture.

Example 2: Construction of rAAV vector backbone (pTR-RRR).

[000249] The vector was constructed using parent plasmid p21NewHP and synthetic oligonucleotides encoding Hammerhead ribozyme (Rz) and RNAi targeting for GFP reporter gene. The respective nucleotide sequences are shown below.

Hammerhead Rz		GFP RNAi	
<i>HindIII</i>	<i>HindIII</i>	<i>XhoI</i>	
A-AGCTTCTGAT GAGCCGTTTCG CGGCGAAACT GCGCCTCCCG CAGTCAAGCT TGGCGATGCC ACCTACGGCA AGCTCGAGCT (SEQ			
ID NO: 1)			
T TCGAAGACTA CTCGGCAAGC GCGCTTTGA CGCGGAGGGC GTCAGTTCGA ACCGCTACGG TGGATGCCGT TCGAGCTCGA (SEQ			
ID NO: 2)			
	Oligos overlap	Stem	Loop
<i>SpeI</i>	Hairpin Rz from p21NewHP		
TGCCGTAGGT GGCATCGCCA CTAGTACAGT CCTGTTTCCT CCAACAGAG AAGTACACCAGAGA (SEQ ID NO: 3)			
ACGGCATCCA CCGTAGCGGT GATCATGTCA GGACAAAGGA GGTGTGTCTC TTCATGTGGTCTCT (SEQ ID NO: 4)			
Stem			

[000250] The oligonucleotides used to assemble the fragment are shown below:

HHTop 5'-AGCTTCTGATGAGCCGTTTCGCGGCGAAACTGCGCCTCCCGCAGT-3' (SEQ ID NO: 5)

HHBottom 5'-CGCCAAGCTTGACTGCGGGAGGCGCAGTTTCGCCGGAACGGCTCATCAGA-3' (SEQ ID NO: 6)

RNAiTop 5'-CAAGCTTGGCGATGCCACCTACGGCAAGCTCGAGCTTGCCGTAGGTGGCATCGCCA-3' (SEQ ID NO: 7)

RNAiBottom 5'-CTAGTGGCGATGCCACCTACGGCAAGCTCGAGCTTGCCGTAGGTGGCAT-3' (SEQ ID NO: 8)

[000251] Oligonucleotides HHBottom and RNAiTop were phosphorylated and annealed to their respective complimentary counterparts (HHTop and RNAiBottom). After annealing, two oligonucleotides were ligated using T4 ligase; the ligation product was gel-purified, phosphorylated and cloned into p21NewHP digested with *HindIII* and *SpeI*. Subsequently, the upstream *HindIII* restriction site was removed by site directed mutagenesis, converting the 5' most A residue to a G. This left a single *HindIII* site for the insertion of other hairpin coding regions downstream of the hammerhead ribozyme.

[000252] An AAV vector comprising an siRNA specific for tyrosine hydroxylase and the green was used to transduce rat brain substantia nigra. Figures 5A-5G show the results from such an experiment. Figure 5A is a low magnification image of transduced rat brain substantia nigra. Figure 5B shows an image of transduced cells fluorescing green due to the GFP gene present in the AAV vector that also expresses an shRNA cassette specific for tyrosine hydroxylase expressed from a pol II promoter (CBA). Figure 5C shows that the red fluorescence is from immunoreactive tyrosine hydroxylase (TH), a bio-marker for all dopaminergic neurons in the nigro-striatal tract. Figures 5D to 5F are higher magnification images. The red arrows (dark colored arrows) indicate a cell highly transduced with AAV and expressing no TH. The yellow arrows (light colored arrows) indicate a partially transduced cell that produces a full amount of TH. Figure 5G is an image showing the effects of AAV transduction with a vector expressing the same siRNA from an H1 promoter with no cassette. This virus preparation had a higher titer and no TH-producing (red) cells were observed).

What is claimed is:

1. A composition comprising a vector expressing a small hairpin RNA which inhibits accumulation, transcription or translation of an intracellular RNA wherein the RNA is an mRNA or a non-coding RNA.
2. The composition of claim 1, wherein the small hairpin RNA comprises hammerhead and hairpin ribozymes flanking a cloning site of a hairpin structure.
3. The composition of claim 1, wherein desired sequences of the RNA hairpin are complementary to a target RNA sequence.
4. The composition of claim 1, wherein the formation of the hairpin RNA is autocatalyzed by a ribozyme.
5. A vector encoding a transcribed isolated RNA molecule, comprising a desired RNA portion, wherein said desired RNA portion is present between a 3' region and a 5' region, wherein said 3' region and said 5' region form an intramolecular stem with each other comprising at least about 15 base pairs, and wherein the RNA molecule is transcribed by an RNA polymerase.
6. The vector of claim 5, wherein the RNA polymerase is RNA polymerase II and the vector is an adenovirus-associated vector.
7. The vector of claim 5, wherein the vector further comprises regulated and/or tissue specific promoters operably linked to the desired RNA.
8. The vector of claim 5, wherein the vector further comprises signal sequences for intracellular trafficking of the RNA molecule.
9. An expression cassette comprising a hammerhead and hairpin ribozyme flanking a cloning site of a hairpin structure wherein a hairpin RNA is of a desired sequence, depending on the target nucleic acid molecule of RNA interference.

10. The expression cassette of claim 9, wherein the ribozymes further comprise an autocatalytic oligonucleotide sequence.

11. The expression cassette of claim 9, wherein the hairpin RNA comprises signal sequences for intracellular targeting of the siRNA and is under control of regulatable and tissue specific promoters.

12. A method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, the method comprising:

targeting the desired nucleic acid molecule with an oligonucleotide; and,
enzymatically degrading the target nucleic acid molecule; thereby,
inhibiting transcription of the target nucleic acid molecule.

13. The method of claim 12, wherein the oligonucleotide targets desired nucleic acid molecules encoded by a wild type gene sequence and any alleles or variants thereof.

14. The method of claim 12, wherein the oligonucleotide targets single-stranded RNA targets.

15. The method of claim 12, wherein the oligonucleotide targets single- and/or double-stranded RNA target molecules.

16. The method of claim 12, wherein the oligonucleotide targets messenger RNA and/or RNA secondary and tertiary structures.

17. The method of claim 12, wherein the oligonucleotide targets genomic molecules as well as episomal structures.

18. The method of claim 12, wherein the oligonucleotide enzymatic activity leads to RNA degradation.

19. The method of claim 12, wherein the oligonucleotide inhibits transcription or translation *in vitro* or *in vivo* of a target nucleic acid molecule.

20. The method of claim 12, wherein the oligonucleotide selectively inhibits replication and/or transcription of a cell comprising the target nucleic acid molecule.

21. The method of claim 12, wherein the target nucleic acid molecule in a cell is expressed in a disease state or is a foreign nucleic acid molecule.

22. The method of claim 12, wherein the disease state is cancer.

23. The method of claim 12, wherein the foreign nucleic acid molecule is from an infectious disease organism.

24. The method of claim 12, wherein the infectious disease organism is virus, protozoa or fungi.

25. The method of claims 12, wherein the siRNA comprises modified base units.

26. The method of claim 25, wherein the modified bases comprise phosphorthiorate, methylphosphonate, peptide nucleic acids, and/or LNA molecules.

27. The method of claim 25, wherein the oligonucleotide comprises about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or comprises only modified base units.

28. A method for selectively treating cells comprising an infectious disease organism, comprising:

administering to the cells an oligonucleotide sequence that is complementary to a target nucleic acid molecule of an infectious disease organism, the cells comprising an oligonucleotide sequence of an infectious disease organism;

targeting a desired nucleic acid sequence wherein the oligonucleotide is at least about 80% complementary to the target nucleic acid sequence; and,

degrading a target nucleic acid sequence, thereby,

inhibiting accumulation and/or translation of the target nucleic acid molecule.

29. The method of claim 28, wherein the cells are mammalian or plant cells.

30. The method of claim 28, wherein the cells are infected with a virus, protozoa or fungi.
31. The method of claim 28, wherein the cells are in any one of G1, S, M, or G2 stage of a cell cycle.
32. The method of claim 28, wherein the oligonucleotide targets a wild type infectious disease organisms' gene sequence and any alleles or variants thereof.
33. The method of claim 28, wherein the oligonucleotide targets single-stranded RNA targets.
34. The method of claim 28, wherein the oligonucleotide targets double-stranded RNA molecules as well as messenger RNA and/or RNA secondary structures.
35. The method of claim 28, wherein the oligonucleotide targets genomic target molecules as well as episomal structures.
36. The method of claim 28, wherein the oligonucleotide comprises modified base units.
37. The method of claim 28, wherein the modified bases comprise phosphorthiorate, methylphosphonate, peptide nucleic acids, and/or LNA molecules.
38. The method of claim 28, wherein the oligonucleotide comprises about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or comprises only modified base units.
39. A method of treating a mammal suffering from or susceptible to an infectious disease or cancer, the method comprising:
administering to the mammal a therapeutically effective amount of an oligonucleotide comprising a ribozyme-siRNA expression cassette wherein the cassette comprises a hammer head ribozyme, an RNA specific for a target nucleic acid molecule and a hairpin ribozyme.

40. The method of claim 39, wherein the infectious disease is caused by or associated with a virus, bacteria, protozoa or fungi.

41. The method of claim 39, wherein the infectious agent is present in any tissue or organ of a mammal.

42. The method of claim 39, wherein the administered oligonucleotide targets messenger RNA of the target gene to inhibit expression thereof.

43. The method of claim 39, wherein administering the oligonucleotide results in inhibition of target gene expression.

44. The method of claim 39, wherein the ribozyme-siRNA expression cassette is encoded by a vector.

45. A composition comprising a ribozyme-siRNA expression cassette wherein the cassette comprises a hammer head ribozyme, an RNA specific for tyrosine hydroxylase and a hairpin ribozyme.

46. The composition of claim 45, wherein the tyrosine hydroxylase specific RNA is flanked in a 5' region by the hammerhead ribozyme and the hairpin ribozyme in a 3' region to said tyrosine hydroxylase RNA.

47. The composition of claim 45, wherein the tyrosine hydroxylase specific RNA is flanked in a 5' region by the hairpin ribozyme and the hammerhead ribozyme in a 3' region to said tyrosine hydroxylase RNA.

48. The composition of claim 45, wherein the hairpin ribozyme comprises about six nucleotides at its 5' region and about nine nucleotides at its 3' end.

49. The composition of claim 45, wherein the tyrosine hydroxylase specific RNA is present between a 3' region and a 5' region, wherein the 3' region and the 5' region form an intramolecular stem with each other comprising at least 15 base pairs.

50. The composition of claim 45, wherein the tyrosine hydroxylase specific RNA is transcribed by a pol II promoter system.

51. The composition of claim 45, wherein the ribozyme-siRNA expression cassette is identified by SEQ ID NO's: 1 through 4.

52. The composition of claim 45, wherein the ribozyme-siRNA expression cassette is constructed using nucleic acid sequences identified by SEQ ID NO's: 5 through 8.

53. A cell in culture comprising a vector encoding a transcribed RNA molecule comprising a desired RNA portion, wherein said desired RNA portion is present between a 3' region and a 5' region, wherein said 3' region and said 5' region form an intramolecular stem with each other comprising at least about 15 base pairs and the RNA molecule is transcribed by RNA pol II.

54. The cell in culture of claim 53, wherein the cell comprises a nucleic acid molecule identified by SEQ ID NO's: 1 through 4.

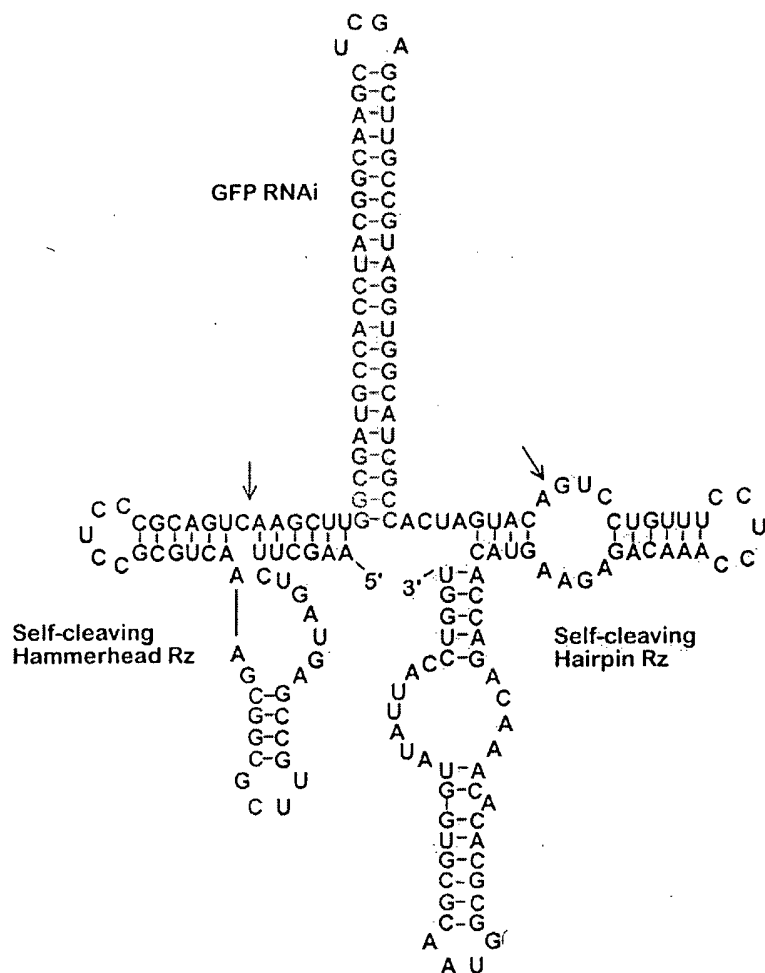


FIGURE 1

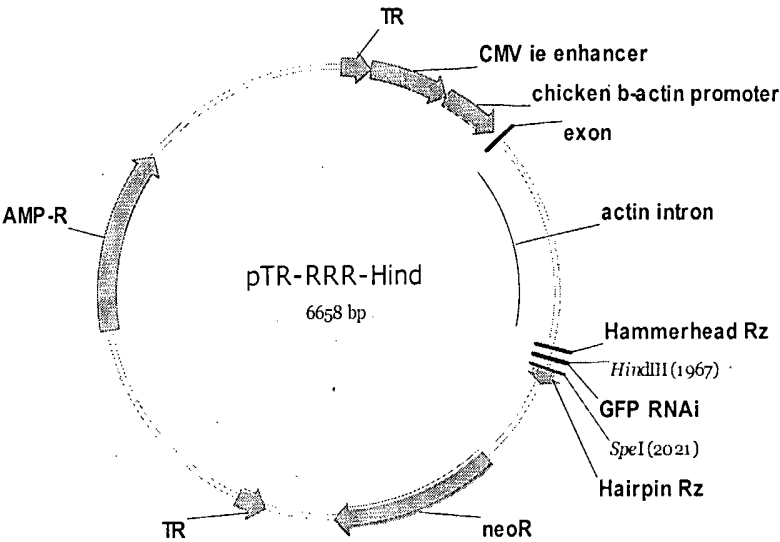


FIGURE 2

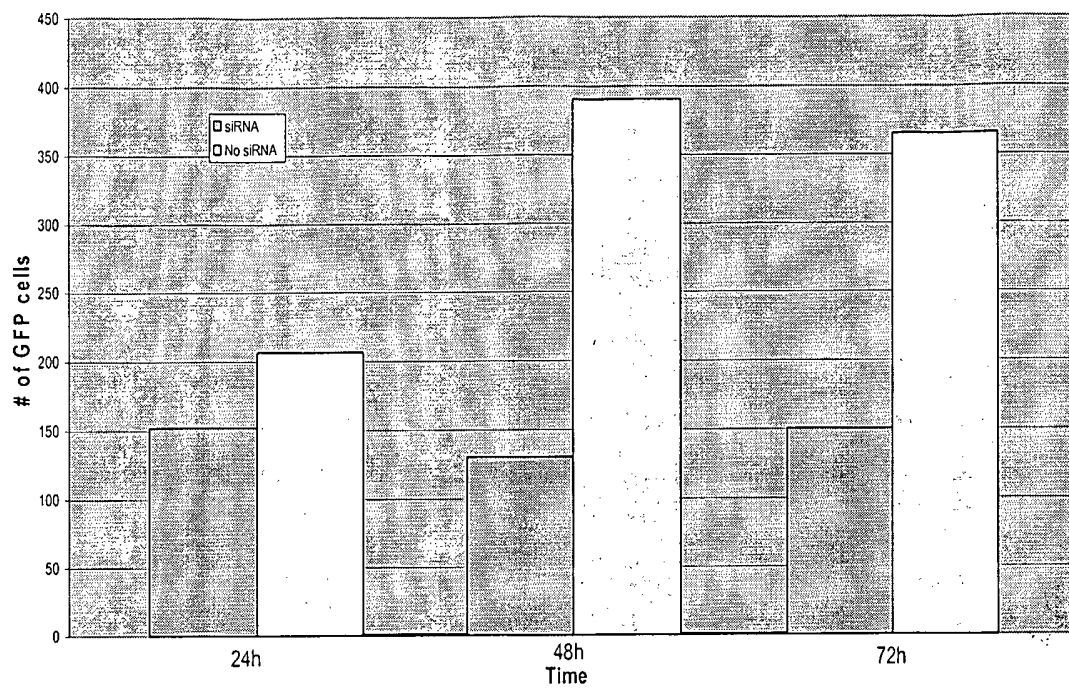


FIGURE 3

Tyrosine hydroxylase staining of sections of rat brain
Both hemispheres TH-siRNA treated GFP siRNA treated

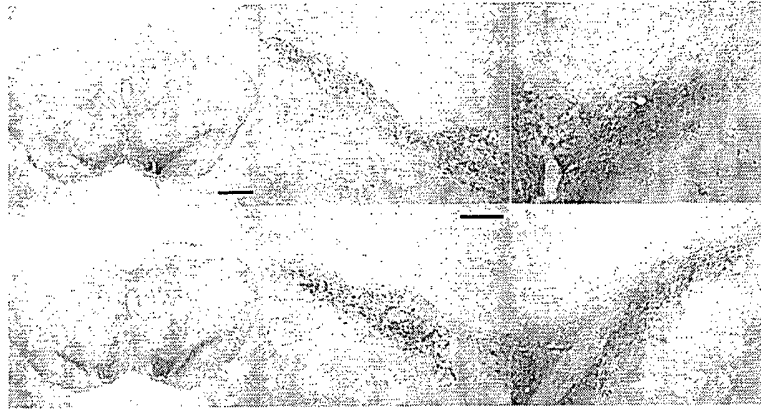


FIGURE 4



FIGURE 5