OPTIMIZED MINIZYMES AND MINIRIBOZYMES AND USES THEREOF

Throughout this application various references are cited in bracket by author and publication year. The full citations are listed alphabetically and may be found immediately preceding the claims. These publications are hereby incorporated by reference into the present application.

Background of the Invention

Several types of ribozymes have been identified in living organisms. One of the first ribozymes to show catalytic turnover was the RNA moiety of ribonuclease P. Ribonuclease P (RNase P) cleaves precursor tRNAs (pre-tRNAs) at their 5' ends to give the mature 5'-termini of tRNAs. In Escherichia coli and Bacillus subtilis, the RNase P holoenzyme is composed of one basic protein subunit of approximate M_r 14,000 (119 amino acids) and one single stranded RNA molecule of 377 and 401 nucleotides, respectively [Baer, 1990; Altman 1987; Waugh, 1989; Pace, 1990; Nichols, 1988]. Another early ribozyme to show cleavage was the L-19 intervening sequence (IVS) from tetrahymena. The 413 nucleotide intervening sequence (IVS) in the nuclear rRNA precursor from Tetrahymena thermophila can be excised and the two exons ligated in the complete absence of any protein [Kruger, 1982; Cech, 1981]. Unique to this class of self-splicing reaction is the requirement of a guanosine or 5' guanosine nucleotide cofactor. The hammerhead, which in nature undergoes a self-cleavage reaction, constitutes a third class of ribozymes. A number of plant pathogenic RNAs [Symons, 1989; Symons, 1990; Bruening, 1989; Bruening 1990], one animal viral RNA [Taylor, 1990] and a transcript from satellite II of DNA of the newt [Epstein, 1987; Epstein

1989] and from a Neurospora DNA plasmid [Saville, 1990] undergo a site specific self-cleavage reaction in vitro to produce cleavage fragments with a 2',3'-cyclic phosphate and a 5'-hydroxyl group. This reaction is unlike RNase P RNA cleavage of pre-tRNAs, where the internucleotide bond undergoes a phosphoryl transfer reaction in the presence of Mg^{++} or other divalent cations. Metal cations may be essential to RNA catalysis [Pyle, 1993]. Other reactions documented to date show that ribozymes can catalyze the cleavage of DNA [Robertson, 1990; Herschlag 1990], the replication of RNA strands [Green, 1992], the opening of 2'-3'-cyclic phosphate rings [Pan, 1992], as well as react with phosphate monoesters [Zaug, 1986] and carbon centers [Noller, 1992; Piccirilli, 1992]. Finally, ribozymes with new kinds of catalytic reactivity are being created through techniques of in vitro selection and evolution [Breaker and Joyce, 1994; Szostak, 1992].

The ability to design a ribozyme to specifically target and cleave any designated RNA sequence has led to much interest in the potential application of hammerhead ribozymes in transgenic plants and in animal health as gene therapy agents or drugs. To improve the ability to treat a disease or target a specific nucleic acid, it is desirable to optimize the ribozyme to achieve the maximum cleavage activity. While much success has been achieved in vitro in targeting and cleaving a number of designated RNA sequences (Saxena and Ackerman, 1990; Lamb and Hay, 1990; Evans, et al., 1992; Mazzolini, et al., 1992; Homann, et al., 1993), there are fewer whole cell examples.

Previous reports have demonstrated that high levels of ribozyme expression are required to achieve reduced accumulation of target sequence in vivo [Cameron and Jennings, 1989; Cotten and Birnsteil, 1989; Sioud and Drilca, 1991; L'Huillier, et al., 1992; Perriman et al., 1993]. Another article suggests a necessity for the target

and ribozyme to be sequestered in the same cellular compartment [Sullenger and Cech, 1993]. These reports demonstrate that hammerhead ribozymes are clearly capable of specific cleavage of a designated target RNA within a biological system.

Summary of the Invention

This invention is directed to improved catalytic compounds, minizymes and miniribozymes, capable of hybridizing with a target RNA to be cleaved. The minizymes and miniribozymes and compositions of the present invention may be used in vitro or in vivo. They may be used as diagnostic or therapeutic agents.

Brief Description of the Figures

Figure 1 shows the CAT Expression (Mean ± SEM (Standard Error of the Mean)) for Different treatments. Each experiment is performed in triplicate.

Figure 2 shows the extent of Alamar Blue Reduction (mean ± SEM) in the same experiments as in Figure 1. Extent of reduction relates to metabolic activity of the cells during the 18 hours post-transfection.

Figure 3 shows the cleavage of the IL-2 substrate at 37°C by minizymes (with cctt and gtttt linkers) and a ribozyme with DNA arms.

Figure 4 shows the numbering scheme of (Hertel 1992) for a hammerhead ribozyme and its substrate (Seq ID No. 1-2).

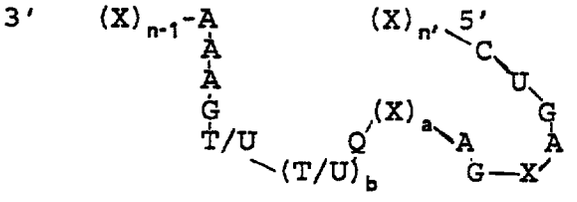
Figure 5 shows time taken for 50% of an 809-nucleotide interleukin-2 transcript to be cleaved by the miniribozyme IL2MGUUUUC (filled circles) and the ribozyme IL2RA (open circles) as a function of temperature.

Sequence of miniribozyme IL2MGUUUUC is 5′ CAAUGCAA CUGAUGA GUUUUC GAAAC AGGa 3′ (SEQ. ID NO. 49) and ribozyme IL2RA is 5′ CAAUGCAA CUGAUGA GUCCUUUUGGAC GAAAC AGGa 3′ (SEQ. ID NO. 50).

The target sequence within the IL2 transcript is 5′ UCCUGUC*UUGCAUUG 3′ (SEQ. ID NO. 51), where * represents the cleavage site which is 82 nucleotides from the 5′ end. Conditions for cleavage experiment: 50 mM Tris.HCl, pH 8, 10 mM $MgCl_2$, 0.2 μM transcript, 1 μM miniribozyme or ribozyme, transcript internally labelled, no heat-denaturing or heat-annealing step was performed.

Detailed Description of the Invention

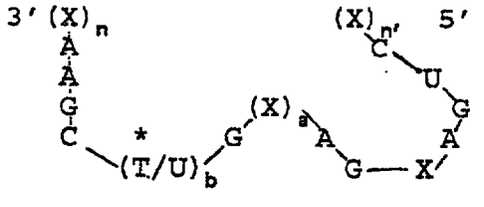
This invention is directed to a compound having the formula (Seq ID No. 3):



wherein each X represents a nucleotide which may be the same or different and Q represents guanidine and may be substituted or modified in its sugar, base or phosphate; wherein each of $(X)_n$ and $(X)_{n'}$ represents an oligonucleotide having a predetermined sequence which is capable of hybridizing with an RNA target sequence to be cleaved; wherein each of n and n' represents an integer which defines the number of nucleotides in the oligonucleotide; wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof; wherein "a" represents an integer which defines a number of nucleotides with the proviso that "a" may be 0 or 1 and if 0, the A located 5' of $(X)_a$ is bonded to the G located 3' of $(X)_a$; and

wherein $(T/U)_b$ represents an oligonucleotide with the proviso that "b" represents an integer which is 3 or 4.

Alternatively, the compound may have the formula (Seq ID No. 4):



In the compounds above, the oligonucleotide 3'-$(X)_n$- is 3'-$(X)_{n-1}$-A- or is 3'-$(X)_{n-2}$-C-A-. Preferably, $(X)_a$ is absent.

The integer "b" of $(T/U)_b$ is preferably equal to 3 or 4. Preferably, $(T/U)_b$ is a $(T)_b$.

The invention is also directed to compositions comprising the compounds above in association with an acceptable carrier; the carrier is preferably a pharmaceutically acceptable carrier.

The invention is also directed to an oligonucleotide transfer vector containing a nucleotide sequence which on transcription gives rise to the compound above. The transfer vector may be a bacterial plasmid, a bacteriophage DNA, a cosmid, an eukaryotic viral DNA, a plant DNA virus, a composite geminivirus, a binary plant expression vector (Ri or Ti) an infective phage particle or a portion thereof. The packaged oligonucleotide transfer vector may contain promoter sequences for RNA polymerase II, human $tRNA^{val}$, plant tRNA, human tRNA, snRNA promoter or RNA polymerase III. The invention also includes a host cell transformed by the transfer vector. The host cell is a prokaryotic host cell, an eukaryotic host cell, an E. coli host cell, a monkey COS host cell, a Chinese hamster ovary host cell, a mammalian host cell, a plant host cell, a plant protoplast host cell, a hematopoietic host cell, a stem cell, a hematopoietic progenitor cell, a lymphoid cell, T-cell, a B-cell, pre-B cell, a CD4+T-cell or a peripheral blood mononuclear cell.

The invention also provides a method of cleaving a target mRNA in a subject which comprises administering to the subject an effective amount of the compound above or a vector capable of expressing the compound. The administration may be topical in an amount between 1 ng and 10 mg. The administration may also be systemic and administered in an amount between 1 ng and 500 μg/kg weight/day. The administration may also be aerosol administration. The invention also provides a method of

cleaving a target mRNA in a host cell which comprises administering to the host cell an effective amount of the compound above.

The compound above may further comprise an antisense nucleic acid which is capable of hybridizing with an RNA target sequence. The compound above may further comprise at least one additional non-naturally occurring oligonucleotide compound which comprises nucleotides whose sequence defines a conserved catalytic region and nucleotides whose sequence is capable of hybridizing with a predetermined target sequence. The additional non-naturally occurring oligonucleotide compound may be a hammerhead ribozyme, a minizyme, a hairpin ribozyme, a hepatitis delta ribozyme, an RNAase P ribozyme or a combination thereof. See for example hammerhead ribozyme Haseloff et al. U.S. Patent No. 5,254,678, issued Oct. 18, 1993; Jennings U.S. Patent No. 5,298,612, issued Mar. 29, 1994; Group I introns, Cech et al. U.S. Patent No. 4,740,463, issued April 26, 1988; Altman et al. U.S. Patent No. 5,168,053, issued Dec. 1, 1992 or PCT International Publication No WO 92/03566), hepatitis delta ribozymes (e.g. Blumenfeld et al. PCT International Application No. WO/90/05157) and hairpin ribozymes (European Patent Application No. EP 360,257, Hampel et al. Nuc. Acids Res. (1990) 18:299-304).

Preferred cleavage sites in the target RNA have the sequence "XUX", preferably GUC, GUU, GUA, UUA and UUC. By way of example, suitable reaction conditions may comprise a temperature from about 4 degree(s) C. to about 60 degree(s) C., preferably from about 10 degree(s) to 45 degree(s) C., more preferably from about 20 degree(s) to 43 degree(s) C., pH from about 6.0 to about 9.0 and concentration of divalent cation (such as Mg^{2+}) from about 1 to about 100 mM (preferably 1 to 20 mM). The nucleotides of the sequences $(X)_n$ and $(X)_{n'}$ of the compounds above may be of any number and sequence sufficient to enable hybridization with the

nucleotides in the target RNA, as described herein. Ribozymes containing a small number of nucleotides in each of the groups $(X)_n$ and $(X)_{n'}$ of the compounds above (such as four nucleotides) would generally be incubated at lower temperatures, such as about 20 degree(s) C. to about 25 degree(s) C. to aid hybridizing with the nucleotide sequences in the substrate. The number of nucleotides n and n' in $(X)_n$ and $(X)_{n'}$ are not necessarily equal. The invention is also directed to covalently-linked multiple ribozymes, where each ribozyme is directed to a target sequence which may be the same or different. In addition these compounds may be covalently attached to an antisense molecule which may be 10 to 100 bases in length. Antisense sequences capable of hybridizing to an RNA in a mammal or plant are well known see (Shewmaker et al. U.S. Patent No. 5,107,065, issued April 21, 1992). As the ribozyme acts as an enzyme, showing turnover, the ratio of ribozyme to substrate may vary widely.

A target RNA containing a suitable cleavage site such as XUX site may be incubated with a compound described above. The nucleotide sequences $(X)_n$ and $(X)_{n'}$ of the compounds above are selected to hybridize with their substrate. They may be selected so as to be complementary to nucleotide sequences flanking the cleavage site in the target RNA. On incubation of the ribozyme or ribozyme composition and its substrate, an enzyme/substrate complex is formed as a result of base pairing between corresponding nucleotides in the ribozyme and the substrate. Nucleotide sequences complementary to $(X)_n$ and $(X)_{n'}$ of the compounds above flanking the cleavage site in the substrate may form a double stranded duplex with $(X)_n$ and $(X)_{n'}$ as a result of base pairing, which base pairing is well known in the art [See for example: Sambrook, 1989]. The formation of a double stranded duplex between the nucleotides may be referred to as hybridization [Sambrook, 1989]. The extent of hybridization or duplex formation between the ribozyme and its substrate can be

readily assessed, for example, by labeling one or both components, such as with a radiolabel, and then subjecting the reaction mixture to polyacrylamide gel electrophoresis under non-denaturing conditions [Sambrook, 1989]. If the target is cleaved specifically on incubation with the compound, the compound is active and falls within the scope of this invention. Accordingly, a ribozyme containing substituted or modified nucleotides in the conserved region may be simply tested for endonuclease activity in a routine manner.

As will be readily appreciated by workers in the field to which this invention relates, the cleavage of a target RNA may be readily assessed by various methods well known in the art [See for example: Sambrook, 1989]. Cleavage may, for example, be assessed by running the reaction products (where the substrate is radioactively labeled) on acrylamide, agarose, or other gel systems under denaturing conditions, and then subjecting the gel to autoradiography or other analytical technique to detect cleavage fragments [Sambrook, 1989].

In another embodiment, the invention provides a composition which comprises the compounds above in association with an acceptable carrier.

The invention is also directed to an oligonucleotide transfer vector containing a nucleotide sequence or sequences which on transcription gives rise to the compounds above. The transfer vector may be a bacterial plasmid, a recombinant bacterial plasmid, a bacteriophage DNA, a cosmid, or an eukaryotic viral DNA. The transfer vector may also contain an appropriate transcription promoter sequence such as that for RNA polymerase II, RNA polymerase III, a viral promoter such as SV40 or HIV LTR, a plant promoter such as CaMV S35 or a promoter associated with animal health. The vector may also contain an appropriate

termination sequence. Preferably, the plant or animal promoter is capable of expression in a regulated manner. Such promoter control regions would be regulated by endogenous signals to direct either tissue specific or temporal expression or by externally administered compounds to elicit transcription of downstream sequences. It may also contain sequences to effect integration into the host genome on episomal replication in the host cell.

The invention also provides a host cell transformed by the transfer vector as mentioned above, which may be a prokaryotic host cell or an eukaryotic host cell e.g. yeast cell or yeast protoplast, E. coli host cell, a monkey host cell (e.g. COS), a Chinese hamster ovary host cell, a mammalian host cell, a plant host cell, or a plant protoplast host cell.

In one embodiment, there is provided a packaged oligonucleotide transfer vector, as mentioned hereinabove, which is a plant virus, a composite mammalian virus, a geminivirus, a Ti or Ri plasmid, an infective phage particle or portion thereof.

In another embodiment, the composition, as discussed above, is in association with an acceptable carrier. This invention also provides a composition as discussed hereinabove wherein the oligonucleotide is an oligoribonucleotide or an RNA-DNA hybrid molecule comprising nucleotides which may be substituted or modified in their sugar, base or phosphate group. It is preferred that the oligonucleotide be an oligoribonucleotide or a hybrid RNA-DNA molecule. However, other substitutions or modifications in the nucleotide are possible providing that endonuclease activity is not lost. Such derivatives or modifications are described below.

The nucleotides may be in the form of deoxyribonucleotides, ribonucleotides, deoxyribonucleotide ribonucleotide hybrids, or derivatives thereof as herein described. The flanking sequences $(X)_n$ and $(X)_{n'}$ may be chosen to optimize stability of the ribozyme from degradation. For example, deoxyribonucleotides are resistant to the action of ribonucleases. Modified bases, sugars or phosphate linkages of nucleotides, such as phosphoramidate, or phosphorothioate linkages in the sugar phosphate chain of X_n and $X_{n'}$, may also provide resistance to nuclease attack. Binding affinity may also be optimized in particular circumstances, by providing nucleotides solely in the form of ribonucleotides, deoxyribonucleotides, or combinations thereof. In some circumstances it may be necessary to optimize the composition of the sequences $(X)_n$ and $(X)_{n'}$, to maximize target RNA cleavage. The cleavage activity of ribozymes having flanking nucleotide sequences which hybridize to target sequences and which are comprised wholly of deoxyribonucleotides may, in some circumstances, have reduced activity. In such circumstances optimization may involve providing a mixture of deoxyribonucleotides and ribonucleotides in the nucleotide sequences $(X)_n$ and $(X)_{n'}$. For example, nucleotides in the ribozyme which are proximal to the cleavage site in a target RNA may be in the form of ribonucleotides.

The respective 3′ and 5′ termini of the sequences $(X)_n$ and $(X)_{n'}$, or alternatively the 3′ and 5′ end termini of the ribozyme, may be modified to stabilize the ribozyme from degradation. For example, blocking groups may be added to prevent terminal nuclease attack, in particular 3′-5′ progressive exonuclease activity. By way of example, blocking groups may be selected from substituted or unsubstituted alkyl, substituted or unsubstituted phenyl, substituted or unsubstituted alkanoyl. Substituents may be selected from C_1 - C_5 alkyl; halogens such as F, Cl or Br; hydroxy; amino; C_1 - C_5 alkoxy and the like. Alternatively,

nucleotide analogues such as phosphorothioates, methylphosphonates or phosphoramidates or nucleoside derivatives (such as alpha - anomer of the ribose moiety) which are resistant to nuclease attack may be employed as terminal blocking groups. The blocking group may be an inverted linkage such as a 3′ 3′ thymidine linkage or a 5′ 5′ pyrophosphate linkage as in the guanosine cap.

Alternatively, groups which alter the susceptibility of the ribozyme molecule to other nucleases may be inserted into the 3′ and/or 5′ end of the ribozyme. For example, 9-amino-acridine attached to the ribozyme may act as a terminal blocking group to generate resistance to nuclease attack on the ribozyme molecules and/or as an intercalating agent to aid endonucleolytic activity. It will be readily appreciated that a variety of other chemical groups, e.g. spermine or spermidine could be used in a related manner.

It is also possible to stabilize the ribozyme from degradation by embedding it in an RNA molecule. These molecules can be produced either in vitro or in vivo by DNA coding sequences being operably linked to transcriptional control sequences as appropriate. Examples of RNA molecules into which ribozymes could be inserted may include, but are not limited to, tRNA, mRNA, rRNA, snRNA or other RNA molecules. In addition, the ribozyme may be inserted into an engineered stable stem loop structure. The compound may also be coupled with other stabilizing structures such as a transcription terminator on the 3′ end such as the T7 terminator, ρ-independent terminator, cry element (Gelfand et al. U.S. Patent No. 4,666,848, issued May 19, 1987) or the TrpE terminator. Furthermore, sequences such as the poly(A) addition signal AATAAA may be added. In addition, strategies involving changing the length of the 3′ non-coding region may be used (see Gillies, U.S. Patent No. 5,149,635, issued September 22, 1992). Alternatively, a stabilizing sequence or protein binding domain such as

Sioud, PCT International application WO 94/10301 may be used. Further, it is possible to insert the compound into a DNA molecule as well.

The compounds of this invention may be covalently or non-covalently associated with affinity agents such as proteins, steroids, hormones, lipids, nucleic acid sequences, intercalating molecules (such as acridine derivatives, for example 9-amino acridine) or the like to modify binding affinity for a substrate nucleotide sequence or increase affinity for target cells, or localization in cellular compartments or the like. For example, the ribozymes of the present invention may be associated with RNA binding peptides or proteins which may assist in bringing the ribozyme into juxtaposition with a target nucleic acid such that hybridization and cleavage of the target sequence may take place. Nucleotide sequences may be added to the respective 3' and 5' termini of the sequences $(X)_n$ and $(X)_{n'}$ or alternatively the 3' and 5' end termini of the ribozyme to increase affinity for substrates. Such additional nucleotide sequences may form triple helices with target sequences [Strobel, 1991] which may enable interaction with an intramolecularly folded substrate. Alternatively, modified bases (non-natural or modified bases as described in Principles of Nucleic Acid Structure [Saenger, 1984]) within the additional nucleotide sequences may be used that will associate with either single stranded or duplex DNA generating base pair, triplet, or quadruplet, interactions with nucleotides in the substrate. Suitable bases would include inosine, 5-methylcytosine, 5-bromouracil and other such bases as are well known in the art, as described, for example, in Principles of Nucleic Acid Structure [Saenger, 1984].

The compounds of this invention may be produced by nucleotide synthetic techniques which are known in the art, and described for example by Carruthers et al., Foehler et

al. and Sproat et al. [Carruthers, 1987; Foehler, 1986; Sproat, 1984]. Generally, such synthetic procedures involve the sequential coupling of activated and protected nucleotide bases to give a protected nucleotide chain, whereafter protecting groups may be removed by suitable treatment. Preferably the compounds will be synthesized on an automated synthesizer such as those made by Applied Biosystems (a Division of Perkin Elmer), Pharmacia or Millipore. Alternatively, the ribozymes in accordance with this invention may be produced by transcription of nucleotide sequences encoding said ribozymes in host-cells or in cell free systems utilizing enzymes such as T3, SP6 or T7 RNA-polymerase.

In addition to being synthesized chemically, ribozymes with modified nucleotides may be synthesized enzymatically. The phosphodiester bonds of RNA can be replaced by phosphorothioate linkages by in vitro transcription using nucleoside α-phosphorthiotriphosphates. T7 RNA polymerase specifically incorporates the Sp isomer of α-phosphorthiotriphosphate with inversion of configuration to produce the Rp isomer of the phosphorothioate linkage. The methods to produce transcripts fully substituted with phosphorothioate linkages adjacent to a given nucleotide, or to produce partially substituted transcripts containing approximately one phosphorothioate linkage. The methods to produce transcripts fully substituted with phosphorothioate linkages adjacent to a given nucleotide, or to produce partially substituted transcripts containing approximately one phosphorothioate linkage per molecule, are described by Ruffner and Uhlenbeck (1990). Conrad et al. (1995) describe methods of using T7 RNA polymerase to produce chimeric transcripts containing ribonucleotides and deoxyribonucleotides (with and without phosphorothioate linkages), and also ribonucleotides and 2'-O-methylnucleotides (with and without phosphorothioate linkages). These methods have been shown to produce

transcripts containing up to 50% deoxyribonucleotides, and up to 58% 2'-O-methylnucleotides. Aurup et al (1992) describe methods for using T7 polymerase to produce transcripts containing 2'-fluoro-2'-deoxyuridine, 2'-fluoro-2'-deoxycytidine, and 2'-amino-2'deoxyuridine. (Aurup, 1992; Conrad, 1995; Ruffner, 1990) Further means for producing the ribozymes of this invention are further discussed below [Sambrook, 1989].

Nucleotides represented in the compounds above comprise a sugar, base, and a monophosphate group or a phosphodiester linkage. Accordingly, nucleotide derivatives or modifications may be made at the level of the sugar, base, monophosphate groupings or phosphodiester linkages. It is preferred that the nucleotides in the compounds above be ribonucleotides or RNA/DNA hybrids, however, other substitutions or modifications in the nucleotide are possible providing that endonuclease activity is not lost.

In one aspect of this invention, the sugar of the nucleotide may be a ribose or a deoxyribose such that the nucleotide is either a ribonucleotide or a deoxyribonucleotide, respectively. Furthermore, the sugar moiety of the nucleotide may be modified according to well known methods in the art [See for example: Saenger, 1984; Sober, 1970]. This invention embraces various modifications to the sugar moiety of nucleotides as long as such modifications do not abolish cleavage activity of the ribozyme. Examples of modified sugars include replacement of secondary hydroxyl groups with halogen, amino or azido groups; 2'-alkylation; conformational variants such as the O2'-hydroxyl being cis-oriented to the glycosyl C_1' -N link to provide arabinonucleosides, and conformational isomers at carbon C_1' to give alpha -nucleosides, and the like. In addition, the invention is directed to compounds with a substituted 2' hydroxyl such as 2' O-allyl, or 2' O-methyl. Alternatively,

the carbon backbone of the sugar may be substituted such as in 2' C-allyl.

Accordingly, the base of the nucleotide may be adenine, 2-amino adenine, cytosine, guanine, hypoxanthine, inosine, methyl cytosine, thymine, xanthine, uracil, or other methylated bases.

Nucleotide bases, deoxynucleotide bases, and ribonucleotide bases are well known in the art and are described, for example in Principles of Nucleic Acid Structure [Saenger, 1984]. Furthermore, nucleotide, ribonucleotide, and deoxyribonucleotide derivatives, substitutions and/or modifications are well known in the art [See for example: Saenger, 1984; Sober, 1970], and these may be incorporated in the ribozyme made with the proviso that endonuclease activity of the ribozyme is not lost. As mentioned previously, endoribonuclease activity may be readily and routinely assessed.

In addition, a large number of modified bases are found in nature, and a wide range of modified bases have been synthetically produced [See for example: Saenger, 1984; Sober, 1970]. For example, amino groups and ring nitrogens may be alkylated, such as alkylation of ring nitrogen atoms or carbon atoms such as N_1 and N_7 of guanine and C_5 of cytosine; substitution of keto by thioketo groups; saturation of carbon-carbon double bonds, and introduction of a C-glycosyl link in pseudouridine. Examples of thioketo derivatives are 6-mercaptopurine and 6-mercaptoguanine. Bases may be substituted with various groups, such as halogen, hydroxy, amine, alkyl, azido, nitro, phenyl and the like. The phosphate moiety of nucleotides or the phosphodiester linkages of oligonucleotides are also subject to derivatization or modifications, which are well known in the art. For example, replacement of oxygen with nitrogen, sulphur or carbon gives phosphoramidates,

(phosphorothioates, phosphorodithioates) and phosphonates, respectively. Substitutions of oxygen with nitrogen, sulphur or carbon derivatives may be made in bridging or non bridging positions. It has been well established from work involving antisense oligonucleotides [Uhlman, 1990] that phosphodiester and phosphorothioate derivatives may efficiently enter cells (particularly when of short length), possibly due to association with a cellular receptor. Methylphosphonates are probably readily taken up by cells by virtue of their electrical neutrality.

A further aspect of the invention provides alternative linkages such as an amide, a sulfonamide, a hydroxylamine, a formacetal, a 3'-thioformacetal, a sulfide, or an ethylene glycol function to replace the conventional phosphodiester linkage. These modifications may increase resistance towards cellular nucleases and/or improved pharmacokinetics.

Information on Synthesis of Protected Nucleotides and their Incorporation into Modified Ribozymes.

POSSIBLE NUCLEOTIDE
Modifications

Sugar Modifications may be 2' fluoro, 2' amino, 2' O-allyl, 2' C-allyl, 2' O-methyl, 2' O-alkyl, 4'-thio-ribose, α-anomer, arabinose, other sugars, or non-circular analogues.

Phosphate Modifications may be phosphorothioate (non-bridging), phosphorodithioate (non bridging), 3' bridging phosphorothioate, 5' briding phosphorothioate, phosphoramidates, 3' bridging phosphoramidate, 5' bridging phosphoramidate, methyl phosphonate, other alky phosphonates or phosphate triesters.

The phosphodiester linkage may be replaced by an amide, carbamate, thiocarbamate, urea, amine, hydroxylamine, formacetal, thioformacetal, allyl ether, allyl, ether, thioether, or PNA (peptide nucleic acid) linkage.

Modifications in base may be purine, 2,6-diaminopurine, 2-aminopurine, O^6-methylguanosine, 5-alkenylpyrimidines, 5-propyne, inosine, 5-methylcytosine, pseudouridine, abasic (ribose or deoxyribose).

Some nucleotides may be replaced with the following chemical linkers: 1,3-propane-diol, alkane-diols, or various polymers of (ethyleneglycol, tetraethylene glycol, hexaethyleneglycol).

Other Modifications to the 3' end may be selected from: 3'-3' inverted linkage (inverted diester or inverted phosphoramidate). 3'-3' linked abasic ribose, or end-capped (methoxyethylamine phosphoramidate).

Modified sugars may be synthesized as follows: 2'-deoxy-2'-fluoro uridine (Sinha, 1984); 2'-deoxy-2' fluoro cytidine (Sinha, 1984); 2'-deoxy-2' fluoroadenosine; synthesis and incorporation into ribozyme (Olsen, 1991); 2'-deoxy-2'-amino uridine and 2'-deoxy-2'-amino cytidine (Heidenreich, 1994); 2'-O-allyl-(uridine or cytidine or adenosine or guanosine) (Available from Boehringer Mannheim, Mannheim, Germany) or (Badger, 1994). 2'-deoxy-2'-C-allyl-ribonucleotides; 2'-O-methyl ribonucleotides see Review: (Sproat, B.S., 1991A) (also Available from Chemgenes, Waltham, MA. or Glen Research, Sterling, VA. other 2'-O-alkyl-ribonucleotides, Synthesis see (Monia, B. P., 1993; Sproat, B. S., 1991B); α-anomer of uridine, cytidine, adenosine and guanosine, see (Debart, F., 1992 and references therein); Other modified sugars, etc.

Arabinose (Garbesi, A., 1993); Hexose-thymidine (Augustyns, K., 1992) and linear analogues of sugars (Hendry, 1994).

Modified phosphates may be synthesized as follows: Phosphorothioate; synthesized by modification of oxidation procedure during phosphoramidite synthesis. Reagents commercially available from Perkin Elmer and others, products are mixture of isomers, some methods available for stereospecific synthesis of phosphorothioate, see ref: (Stec, 1991); Phosphorodithioate; (Eldrup, A. B., 1994; Caruthers, 1991; Beaton, 1991); 3'-bridging phosphorothioate; 5' bridging phosphorothioate; phosphoramidates (non-bridging, oxidize the phosphite triester with solution containing the required amine); (Froehler, B., 1988; Jager, A., 1988; Letsinger, R.L., 1988); 3' bridging phosphoramidate (NH replaces 3' O) (Forms very stable duplexes) (Letsinger, 1992; Gryaznov, S. M., 1995; Chen, J. K., 1995); 5' Bridging Phosphoramidate (NH replaces 5' O; thymidine analogue only, weak binder) (Gryaznov, S. M., 1992); Methylphosphonate (reagents are commercially available; Glen Research or Chemgenes Stereospecific; Rp isomers bind stronger: (Savchenko, 1994; Miller, 1991); 5'-deoxy, 5'-methylphosphonate (Szabo, 1995); Other alkyl-phosphonates (Fathi, 1994A; Fathi, 1994B); Phosphate triesters(Summers, 1986).

Replacements for the Phosphodiester Linkage may be synthesized as follows:
For review see (De Mesmaker, 1995) Amides (Chur,1993; Blommers, 1994; De Mesmaeker, 1993; De Mesmaeker, 1994A; De Mesmaeker, 1994B; Lebreton, 1993; Lebreton, 1994A; Lebreton, 1994B; Idsiak, 1993): Carbamate (Waldner, 1994; Stirchak, 1987; Habus, 1994; Thiocarbamate (Waldner,1995); Ureas (Waldner, 1994) Amines (De Mesmaeker,1994C; Caulfield, 1994); Hydroxylamine (Debart,1992; Vasseur,1992; Formacetal (Matteucci,1990; Jones, 1993)Thioformacetal (Jones,1993); Allyl ether (Cao,1994); Allyl, Ether, Thioether (Cao,

1994); Alkane (De Mesmaeker,1994; PNA A selection of binding and antisense properties (Nielsen, 1993A; Hanvey, 1992; Egholm, 1993; Nielsen, 1993B); PNA Synthesis (Egholm, 1992A; Egholm, 1992B);Prepn of purine PNA monomers and oligos (available commercially from Millipore corporation).

Modified bases may be synthesized as follows:
Purine; synthesis and incorporation into ribozyme (Slim, 1992; Fu,1992; Fu, 1993); 7-deazaGuanosine, synthesis and incorporation into ribozyme (Fu, 1993); Inosine, synthesis and incorporation into ribozyme (Slim,1992; Fu, 1993)7-deazaAdenosine, synthesis and incorporation into ribozyme (Fu, 1992; Seela, 1993). O6-methylguanosine, synthesis and incorporation into ribozyme (Grasby, 1993); 2,6-diaminopurine, synthesis (Sproat, 1991); 2-aminopurine, synthesis and incorporation into ribozyme (Ng, 1994; Tuschl, 1993); Isoguanosine, synthesis and incorporation into ribozyme (Ng, 1994; Tuschl, 1993); Xanthosine, synthesis and incorporation into ribozyme (Tuschl, 1993);6-azathymidine, 6-aza-2'-deoxycytidine, synthesis and incorporation into oligonucleotides (Sanghvi, 1993); 5-alkenylpyrimidines; 5-propyne (Gilead, Froehler); inosine; 5-methylcytosine; pseudouridine; abasic ribose or deoxyribose.

SUMMARY OF NUCLEOTIDE-MODIFICATIONS WHICH HAVE BEEN TESTED IN RIBOZYMES.

Sugars.

Modifications may be made to the 2'OH group of the sugar at all non-conserved nucleotides; modifications tested have been 2'H (DNA), 2'F, 2'amino, 2'-O-allyl, 2'-O-methyl, 2'-C-allyl.

Selected modifications may be made to the 2'OH groups of the conserved nucleotides C3, U4, A6, N7, A9, G12, A13, A14, N15.2.

Modifications cannot be made to the 2'OH groups of G5, G8 and A15.1.

For a ribozyme with good cleavage activity, modifications should not be made to G5, A6, G8, G12, A15.1 (except G12 can be 2'H (DNA)).

Generally, except for modifications at G5, G8 and A15.1, no single modification causes a big reduction in cleavage activity; however, activity decreases as more modifications are included in the ribozyme.

Phosphates.

The phosphate groups of the nonconserved nucleotides may be phosphorothioates (phosphorothioated DNA). Preferably, when non-conserved nucleotides are DNA, only two or three phosphates at the 3' and 5' ends of the ribozyme are phosphorothioates.

The phosphates 5' to the conserved nucleotides C3, U4, G5, G8 and G12, and 3' to A9 and N15.2, may be phosphorothioates; but phosphates 5' to A9, A13 and A14 may not be phosphorothioates.

Conserved nucleotides.

C3

Sugar - 2'-OH group can be modified (except, probably, for 2'amino). 2'H (Yang, 92), 2'F (Pieken, 91; Heidenreich, 92), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are all modifications that permit cleavage.

Possibly cannot have 2'amino modification (several Cs in ribozyme had 2'amino modification which resulted in reduction in activity, and effect is probably due to 2'amino on C3 and/or C15.2) (Pieken, 91).

Phosphate - 5' phosphate can be phosphorothioate (Shimayama, 93)

U4

Sugar - 2'OH group can be modified. 2'H (Yang, 92), 2'F (Pieken, 91; Heidenreich, 92), 2'amino (Pieken, 91), 2'-C-allyl (Usman, 95), 2'-O-allyl (but keep as 2'OH if A6 is 2'-O-allyl) (Paolella, 92) are modifications that permit cleavage.

Phosphate - 5' phosphate can be phosphorothioate (Shimayama, 93)

G5

Base - 2-amino group on G base is essential (cannot be inosine) (Oddi, 90; Fu, 92)

Sugar - cannot make modifications to 2'OH of G5. Cannot have 2'H (Perreault, 90; Perreault, 91; Fu, 92; Williams, 92), 2'amino (Pieken, 91; Williams, 92), 2'-O-methyl (Paolella, 92), 2'F (Williams, 92).

A6

Base - can be purine (i.e. 6-amino group is not essential) (Fu, 92). N7 cannot be C7 in A base (Fu, 92).

Sugar- 2'OH group can be modified. 2'H (Perreault, 90; Olsen, 91; Yang, 92; Fu, 92), 2'F (Olsen, 91), 2'-O-allyl (but only if U4 is 2'OH) (Paolella, 92) are modifications that permit cleavage.

N7

Seems to be a sensitive site for pyrimidine endonucleases; protection achieved if rN is rG or rA (Shimayama, 93).

Sugar - 2'OH group can be modified. 2'H (tested dT) (Yang, 92), 2'F (Pieken, 91; Heidenreich, 92), 2'-amino (Pieken, 91), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are all modifications that permit cleavage.

3' phosphate can be phosphorothioate (has been tested for N = U) (Shimayama, 93).

G8

Sugar - cannot make modifications to 2'OH of G8. Cannot have 2'H (Fu, 92; Williams, 92; Yang, 92), 2'F (Williams, 92), 2'amino (Williams, 92), 2'-O-allyl (Paolella, 92). (Perreault (91) says can have 2'H, but Yang (92) says this site is critical if lots of other conserved nucleotides are DNA.)

Phosphate - 5' phosphate probably can be phosphorothioate (see N7 phosphate).

A9

Sugar - 2'OH group can be modified. 2'H (Olsen, 91; Fu, 92; but Perreault (91) says cannot be 2'H), 2'F (Olsen, 91;

Pieken, 91), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are all modifications that permit cleavage.

Phosphate - 5' phosphate cannot be phosphorothioate (Buzayan, 90; Ruffner, 90). 3' phosphate can be phosphorothioate (Shimayama, 93).

G12

Base - 2-amino group is essential (cannot be inosine) (Slim, 92).

Sugar - 2'OH group can tolerate some modifications. 2'H (Perreault, 91; Yang, 92; Williams, 92), 2'amino (Pieken, 91; Williams, 92) are OK. Cannot be 2'F (Williams, 92), 2'-O-allyl (Paolella, 92).

Phosphate - 5' phosphate can be phosphorothioate (Shimayama, 93).

A13

Base - Can change N7 to C. in A base (Fu, 92). 6-amino group essential (cannot be purine) (Slim, 92).

Sugar - 2'OH group can tolerate some modifications. 2'H (Perreault, 91; Yang, 92), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are modifications that permit cleavage. Cannot have 2'F if each of A13, A14, A15.1 have 2'F (Pieken, 91).

Phosphate - 5' phosphate cannot be phosphorothioate (Ruffner, 90).

A14

Base - Can change N7 to C. (Fu, 92). Can be purine (Slim, 92).

Sugar - 2'OH group can tolerate some modifications. 2'H (Perreault, 91; Yang, 92), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are modifications that permit cleavage. Cannot have 2'F if each of A13, A14, A15.1 have 2'F (Pieken, 91).

Phosphate - 5' phosphate cannot be phosphorothioate (Ruffner, 90).

A15.1

Base - Can change N7 to C. (Fu, 92). 6-amino group essential (cannot be purine) (Slim, 92).

Sugar - Cannot modify 2'OH. Cannot have 2'H (Yang, 92), 2'-O-allyl (Paolella, 92), 2'F (if A13 and A14 also are 2'F) (Pieken, 91).

N15.2

Sugar - selected modifications permit cleavage. 2'F (Pieken, 91; Heidenreich, 92), 2'-O-allyl (Paolella, 92), 2'-O-Methyl (Usman, 95) are modifications that permit cleavage.

Rates are low if 2'H (Yang, 92). Possibly cannot have 2'amino modification (several Cs in ribozyme had 2'amino modification which resulted in reduction in activity, and effect is probably due to C3 and/or C15.2) (Pieken, 91).

3' phosphate can be phosphorothioate (Shimayama, 93)

Modifications at the 3' end of an oligonucleotide or ribozyme.

3'MEA(methoxyethylamine)phosphoramidate in last two (or last) internucleotide linkages; 3'-3' inverted diester linkage or 3'-3' inverted phosphoramidate (Shaw, 91).

3'-3' inverted Thymidine, or 3'-3' linked abasic ribose (Usman, 95).

Any combination of the above listed nucleotide modifications, substitutions, or derivatizations, made at the level of the sugar, base, or monophosphate groupings or phosphophodiester linkages may be made in the compounds provided that endonuclease activity is not lost.

The compounds of this invention may be incorporated and expressed in cells as a part of a DNA or RNA transfer vector, or a combination thereof, for the maintenance, replication and transcription of the ribozyme sequences of this invention.

Nucleotide sequences encoding the compounds of this invention may be integrated into the genome of a eukaryotic or prokaryotic host cell for subsequent expression (for

example as described [Sambrook, 1989]). Genomic integration may be facilitated by transfer vectors which integrate into the host genome. Such vectors may include nucleotide sequences, for example of viral or regulatory origin, which facilitate genomic integration. Methods for the insertion of nucleotide sequences into a host genome are described for example in Sambrook et al. and Hogan et al. [Sambrook, 1989; Hogan, 1986; 1989].

Nucleic acid sequences encoding the ribozymes of this invention integrated into the genome may preferably include promoter and enhancer elements operably linked to the nucleotide sequence encoding the ribozyme of this invention, with an appropriate termination sequence and capable of expressing said ribozyme in a eukaryotic (such as animal or plant cells) or prokaryotic (such as bacteria) host cells. Ideally, the promoter and enhancer elements are designed for expression in a tissue and/or developmentally specific manner.

Additionally, the compounds of the present invention may be prepared by methods known per se in the art for the synthesis of RNA molecules. (For example, according to recommended protocols of Promega, Madison, Wis., USA). In particular, the ribozymes of the invention may be prepared from a corresponding DNA sequence (DNA which on transcription yields a ribozyme, and which may be synthesized according to methods known per se in the art for the synthesis of DNA) operably linked to an RNA polymerase promoter such as a promoter for T3 or T7 polymerase or SP6 RNA polymerase. A DNA sequence corresponding to a ribozyme of the present invention may be ligated into a DNA transfer vector, such as plasmid or bacteriophage DNA. Where the transfer vector contains an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, the ribozyme may be conveniently produced upon incubation with an RNA polymerase. Ribozymes may, therefore, be produced in vitro

by incubation of RNA polymerase with an RNA polymerase promoter operably linked to DNA encoding a ribozyme, in the presence of ribonucleotides and an appropriate buffer. In vivo, prokaryotic or eukaryotic cells (including mammalian, plant and yeast cells) may be transfected with an appropriate transfer vector containing genetic material encoding a ribozyme in accordance with the present invention, operably linked to an RNA polymerase promoter such that the ribozyme is transcribed in the host cell. Transfer vectors may be bacterial plasmids or viral (RNA and DNA) or portion thereof. Nucleotide sequences corresponding to ribozymes are generally placed under the control of strong promoters such as, the lac, SV40 late, SV40 early, metallothionein, or lambda promoters. Particularly useful are promoters regulated in a tissue or a temporal (developmental) specific manner or tightly regulated inducible promoter suitable for gene theory, which may be under the control of exogenous chemicals. The vector may be an adenovirus or an adeno-associated virus. See for example PCT International Publication No. WO 93/03769, "Adenovirus Mediated Transfer of Genes to the Gastrointestinal Tract", U.S. Serial No. 747,371; PCT International Publication No. WO 94/11506, "Adenovirus-Mediated Gene Transfer to Cardiac and Vascular Smooth Muscle," J. Leiden et al., U.S. Serial No. 07/977,496; PCT International Publication No. WO 94/11522, "Vector for the Expression of Therapy-Relevant Genes," U. Stein et al.,PCT International Publication No. WO 94/11524, "Targetable Vector Particles," W. Anderson et al., U.S. Serial No. 973,307;

PCT International Publication No. WO 94/17832, "Targeting and Delivery of Genes and Antiviral Agents into Cells by the Adenovirus Penton," G. Nemerow et al., U.S. Serial Nos. 08/046,159 and 08/015,225. Ribozymes may be directly transcribed in vivo from a transfer vector, or alternatively, may be transcribed as part of a larger RNA molecule. For example, DNA corresponding to ribozyme sequences may be ligated into the 3' end of a reporter gene,

for example, after a translation stop signal. Larger RNA molecules may help to stabilize the ribozyme molecules against nuclease digestion within cells. On translation the reporter gene may give rise to a protein, possibly an enzyme whose presence can be directly assayed.

The compounds of this invention may be involved in gene therapy techniques, where, for example, cells from a human suffering from a disease, such as HIV, are removed from a patient, treated with the ribozyme or transfer vector encoding the ribozyme to inactivate the infectious agent, and then returned to the patient to repopulate a target site with resistant cells, so called ex vivo therapy. In the case of HIV, nucleotide sequences encoding ribozymes of this invention capable of inactivating the HIV virus may be integrated into the genome of lymphocytes or may be expressed by a non-integrating vector such as adenovirus. Such cells would be resistant to HIV infection and the progeny thereof would also confer such resistance.

A transfer vector such as a bacterial plasmid or viral RNA or DNA or portion thereof, encoding one or more of the compounds, may be transfected into cells of an organism in vivo [See for example: Llewellyn, 1987; Hanahan, 1983]. Once inside the cell, the transfer vector in some cases may replicate and be transcribed by cellular polymerases to produce ribozyme RNAs which may have ribozyme sequences of this invention; the ribozyme RNAs produced may then inactivate a desired target RNA. Alternatively, a transfer vector containing one or more ribozyme sequences may be transfected into cells by electroporation, PEG, high velocity particle bombardment or lipofectants, or introduced into cells by way of micromanipulation techniques such as microinjection, such that the transfer vector or a part thereof becomes integrated into the genome of the host cell. Transcription of the integrated genetic material gives rise to ribozymes, which act to inactivate a desired target RNA.

Transfer vectors expressing ribozymes of this invention may be capable of replication in a host cell for stable expression of ribozyme sequences. Alternatively, transfer vectors encoding ribozyme sequences of this invention may be incapable of replication in host cells, and thus may result in transient expression of ribozyme sequences. Methods for the production of DNA and RNA transfer vectors, such as plasmids and viral constructs are well known in the art and are described for example by Sambrook et al. [Sambrook, 1989].

Transfer vectors would generally comprise the nucleotide sequence encoding the ribozyme of this invention, operably linked to a promoter and other regulatory sequences required for expression and optionally replication in prokaryotic and/or eukaryotic cells. Suitable promoters and regulatory sequences for transfer vector maintenance and expression in plant, animal, bacterial, and other cell types are well known in the art and are described for example in Hogan [Hogan, 1986; 1989].

The ribozymes of the present invention have extensive therapeutic and biological applications. For example, disease causing viruses in man and animals may be inactivated by administering to a subject infected with a virus, a ribozyme in accordance with the present invention adapted to hybridize to and cleave specific RNA transcripts of the virus. Such ribozymes may be delivered by parenteral or other means of administration. Alternatively, a subject infected with a disease causing virus may be administered a non-virulent virus such as vaccinia or adenovirus which has been genetically engineered to contain DNA corresponding to a ribozyme operably linked to an RNA promoter, such that the ribozyme is transcribed in the cells of the host animal, transfected with the engineered virus, to effect cleavage and/or inactivation of the target RNA transcript of the disease causing virus.

The ribozymes of the present invention have particular application to viral diseases caused for example, by the herpes simplex virus (HSV) or the AIDS virus (HIV). Further examples of human and animal disease which may be treated with the ribozymes of this invention include psoriasis, cervical preneoplasia, papilloma disease, bacterial and prokaryotic infection, viral infection and neoplastic conditions associated with the production of aberrant RNAs such as occurs in chronic myeloid leukemia. Diseases or infections which may be treated in plants with ribozymes of this invention include fungal infection, bacterial infections (such as Crown-Gall disease) and disease associated with plant viral infection. Of particular interest would be compounds targeting genes associated with male gametophyte development. Examples include PCT International Publication No. WO 92/18625, entitled "Male-Sterile Plants, Method For Obtaining Male-Sterile Plants And Recombinant DNA For Use Therein"; U.S. Patent No. 5,254,802, entitled "Male Sterile Brassica Plants," S. Hoekstra et al.; PCT International Publication No. WO 93/25695, entitled "Maintenance of Male-Sterile Plants," M. Williams et al., claiming the priority of U.S. Serial Nos. 07/970,840 and 07/899,072; PCT International Publication No. WO 94/25593, entitled "Method For Obtaining Male-Sterile Plants" Stiekema et al.; PCT International Publication No. WO 94/29465, entitled "Process For Generating Male Sterile Plants" Dirks et al.

The period of treatment would depend on the particular disease being treated and could be readily determined by a physician or by a plant biologist as appropriate. Generally treatment would continue until the disease being treated was ameliorated.

The ribozymes of the present invention also have particular application to the inactivation of RNA transcripts in bacteria and other prokaryotic cells, plants, animals and

yeast cells. In bacteria, RNA transcripts of, for example, bacteriophage, (which cause bacterial cell death) may be inactivated by transfecting a cell with a DNA transfer vector which is capable of producing a ribozyme in accordance with the present invention which inactivates the phage RNA. Alternatively, the ribozyme itself may be added to and taken up by the bacterial cell to effect cleavage of the phage RNA. Similarly, eukaryotic and prokaryotic cells in culture may, for example, be protected from infection or disease associated with mycoplasma infection, phage infection, fungal infection and the like.

RNA transcripts in plants may be inactivated using ribozymes encoded by a transfer vector such as the Ti plasmid of Agrobacterium tumefaciens. When such vectors are transfected into a plant cell and integrated, the ribozymes are produced under the action of RNA polymerase and may effect cleavage of a specific target RNA sequence. Endogenous gene transcripts in plants, animals or other cell types may be inactivated using the compounds of the present invention. Accordingly, undesirable phenotypes or characteristics may be modulated. It may, for example, be possible using the ribozymes of the present invention to remove stones from fruit or treat diseases in humans which are caused by the production of a deleterious protein, or over production of a particular protein. The compounds described above may be used to effect male sterility by destroying the pollen production in a plant. Furthermore, for the in vivo applications of the ribozymes of this invention in humans, animals, plants, and eukaryotic and prokaryotic cells, such as in phenotypic modification and the treatment of disease, it is necessary to introduce the ribozyme into cells whereafter, cleavage of target RNAs takes place. In vivo applications are highly suitable to the compounds as discussed herein.

Methods for the introduction of RNA and DNA sequences into cells, and the expression of the same in prokaryotic and eukaryotic cells are well known in the art for example as discussed by Cotten and Friedman [Cotten, 1990; Friedman, 1989]. The same widely known methods may be utilized in the present invention.

The compounds of this invention may be incorporated into cells by direct cellular uptake, where the ribozymes of this invention would cross the cell membrane or cell wall from the extracellular environment. Agents may be employed to enhance cellular uptake, such as liposomes or lipophilic vehicles, cell permeability agents, such as dimethylsulfoxide, and the like.

The compounds of the present invention may be combined with pharmaceutically and veterinarally acceptable carriers and excipients which are well known in the art, and include carriers such as water, saline, dextrose and various sugar solutions, fatty acids, liposomes, oils, skin penetrating agents, gel forming agents and the like, as described for example in Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Co., Easton, Pa., Edited by Ostol et al., which is incorporated herein by reference.

Agriculturally acceptable carriers and excipients are well known in the art and include water; surfactants; detergents; particularly biodegradable detergents; talc; inorganic and/or organic nutrient solutions; mineral earths and clays; calcium carbonate; gypsum; calcium sulfate; fertilizers such as ammonium sulfate, ammonium phosphate, urea, carborundum, and agrobacterium tumefaciens; and natural products of vegetable origin such as, for example, grain, meals and flours, bark meals; and the like.

The compounds of this invention may be provided in a composition with one or more anti-viral, anti-fungal,

anti-bacterial, anti-parasitic, anti-protazoan or antihelminthic agents, herbicides, pesticides or the like, for example as described in the Merck Index (1989) 11th Edition, Merck & Co. Inc.

By way of example only, therapeutic compositions of this invention may be directed against Herpes Simplex virus types 1 and 2, psoriasis, cervical preneoplasia, papilloma disease, and bacterial and prokaryotic infection. Such treatments may, for example, involve topical application of ribozyme to the site of disease. For example, in the treatment of Herpes virus lesions, ribozymes may be formulated into a cream containing a concentration of 0.1 nM to 100 mM ribozyme, preferably 1 nM to 1 mM. The cream may then be applied to the site of infection over a 1 to 14 day period in order to cause amelioration of symptoms of the infection. Prior to the final development of topical formulations for the treatment of virus infection, effectiveness and toxicity of the ribozymes and formulations involving them may, for example, be tested on an animal model, such as scarified mouse ear, to which virus particles, such as 2 X 10^6 plaque forming units are added. A titer of infectious virus particles in the ear after treatment can then be determined to investigate effectiveness of treatment, amount of ribozyme required and like considerations. Similar investigations in animal models prior to human trials may also be conducted, for example, in respect of the treatment of psoriasis, papilloma disease, cervical preneoplasia, and in diseases such as HIV infection, bacterial or prokaryotic infection, viral infection and various neoplastic conditions, which neoplastic conditions involve a deleterious RNA species.

Compositions for topical application are generally in the form of creams, where the ribozymes of this invention may be mixed with viscous components. The compounds of this invention may be incorporated into liposomes or other

barrier type preparations to shield the ribozymes from nuclease attack or other degradative agents (such as nucleases and adverse environmental conditions such as UV light).

Compositions may be provided as unit dosages, such as capsules (for example gelatin capsules), tablets, suppositories and the like. Injectable compositions may be in the form of sterile solutions of ribozyme in saline, dextrose or other media. Compositions for oral administration may be in the form of suspensions, solutions, syrups, capsules, tablets and the like. Ribozymes may also be provided in the form of an article for sustained release, impregnated bandages, patches and the like. The compounds of this invention may be embedded in liposomes or biodegradable polymers such as polylactic acid. Pharmaceutical compositions which may be used in this invention are described, for example, in Remington's Pharmaceutical Sciences, see above.

The present invention is further directed to a plant DNA expression cassette comprising a gene sequence flanked by promoter and terminator sequences at its 5'- and 3'ends respectively wherein said genetic sequence on expression provides a ribozyme RNA. The DNA cassette may further be part of a DNA transfer vector suitable for transferring the DNA cassette into a plant cell and insertion into a plant genome. In a most preferred embodiment of the present invention, the DNA cassette is carried by broad host range plasmid and which is capable of transformation into plant cells using Agrobacterium comprising Ti DNA on the left and right borders, a selectable marker for prokaryotes, a selectable marker for eukaryotes, a bacterial origin of replication and optional plant promoters and terminators such as pGA470. The present invention also includes other means of transfer such as genetic bullets (e.g. DNA-coated tungsten particles, high-velocity micro projectile

bombardment) and electroporation amongst others [Maliga, 1993; Bryant, 1992; or Shimamoto, 1989].

The present invention is also directed to a transgenic plant resistant to a virus, its genome containing a sequence which gives rise, on transcription, to the nucleic acid molecule mentioned above. This transgenic plant, including fruits, and seeds thereof, may be from alfalfa, apple, arabidopsis, barley, bean, canola (oilseed rape), cantaloupe, carnation, cassava, casuarina, clover, corn, cotton, courgette, cucumber, eucalyptus, grape, melon, papaya, pepper, potato, rice, rose, snap dragon, soybean, squash, strawberry, sunflower, sweet pepper, tobacco, tomato, walnut, wheat or zucchini. Also included are the plant cells transformed by the above-mentioned transfer vector, as well as a prokaryotic, eukaryotic or yeast, plant or animal cell, comprising a nucleotide sequence which is, or on transcription gives rise to, the nucleic acid molecule.

The present invention will now be illustrated by way of non-limiting Examples only, with reference to the following non-limiting Examples, and Figures.

Example 1

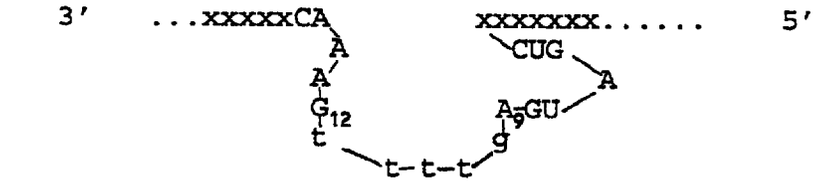
Minizymes containing the deoxyribonucleotides d(GTTTT) and d(GTTTTT) between the conserved nucleotides A_9 and G_{12} offer the following advantages:

(i) These minizymes show fast cleavage rates <u>in vitro</u>. See following data for the test systems Interleukin-2, TAT, CAT, and TNFα.

(ii) The CAT minizyme (CATMgtttt) shows activity against CAT in CHO cells (see Example 2).

(iii) The IL2 minizyme (IL2Mgtttt) shows activity against Interleukin-2 in PBMN cells (see Experiment 3)(Seq ID No. 5).

Minizyme g t t t t



1. Method for determining rates of cleavage (k_{obs}) by minizymes.

In these experiments, conditions are optimized so that the rate-limiting step in the reaction is cleavage of the substrate. The substrate consists of only a small number of nucleotides, in order to prevent strong self-association, and hence substrate association with the minizyme is not rate-limiting. In addition, the minizyme concentration is at least two-fold greater than substrate concentration, which is high enough to ensure all substrate molecules are bound by minizymes. Thus, the measured rate in these experiments should be rate of cleavage of the substrate.

The substrate is labelled on its 5' end with [^{32}P]-phosphate. In general, the minizyme and substrate are heated together in buffer for two minutes at 80°C without magnesium, in

order to denature the nucleic-acid molecules; however, this step has been shown not to be necessary in a number of cases. The cleavage reaction is initiated by adding Mg^{++} to the mixture at 37°C. [$MgCl_2$] = 10mM, [Tris.HCl buffer] = 50 mM, [Minizyme] = 5 μM (typically), [Substrate] = 2 μM (typically), temperature = 37°C, pH 8.2 (for Interleukin-2 and TAT systems) and pH 8.0 (for CAT and TNFα systems). Samples are taken from the reaction mixture at various times, and the reaction is stopped by adding excess EDTA and formamide. The samples are electrophoresed on a polyacrylamide gel containing 7M urea, and the amounts of 5'-product and uncleaved substrate are quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software. Kinetic parameters are obtained by fitting the data for % of product formed (P_t) versus time (t) to the equation

$$P_t = P_\infty - (\exp(-k_{obs}t)P_\Delta)$$

where P_t is the amount of product at time t, P_∞ is the amount of product at $t = \infty$, k_{obs} is the first-order rate-constant for the reaction, and P_Δ is the difference between the percentage of product at $t = \infty$ and $t = 0$. This is a conventional first-order kinetic equation from which k_{obs}, P_∞, and P_Δ are determined by least-squares fitting of the data.

2. Sequences of molecules.

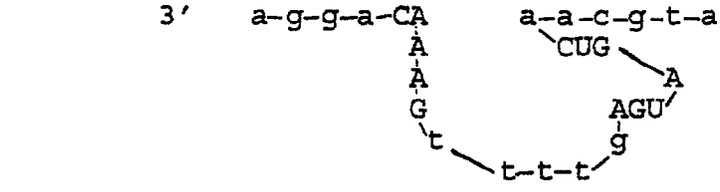
Upper-case letters represent ribonucleotides, lower-case letters represent deoxyribonucleotides.

Interleukin-2 system. (Seq ID No. 6-10)

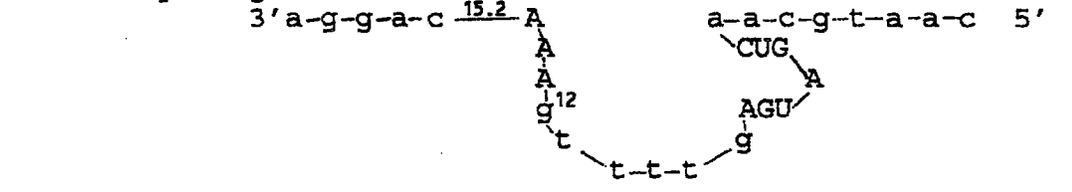
Substrate (15-mer)

5' UCCUGUC UUGCAUUg 3'

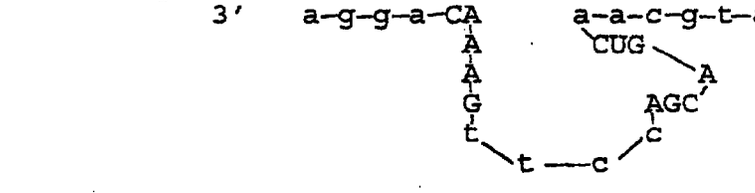
Minizyme g t t t t



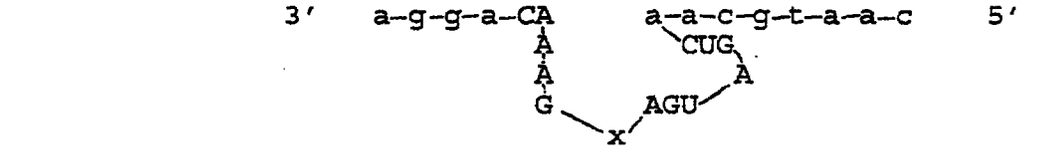
Minizyme $dg^{12}dc^{15.2}$



Minizyme c c t t



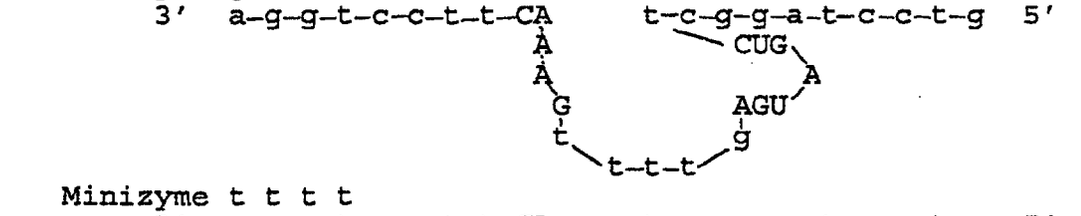
Other interleukin-2 minizymes. "x" represents the deoxyribonucleotides forming the linker between the ribonucleotides. The various sequences of "x" that have been tested appear in the following table (Table 1).



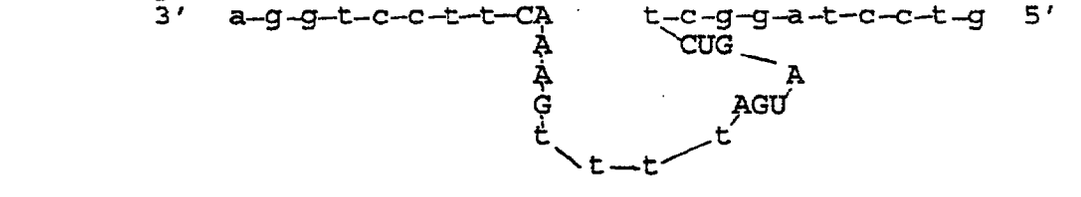
TAT system. (Seq ID No. 11-13)

Substrate (13-mer) 5' GGAAGUC AGCCUa 3'

Minizyme g t t t t



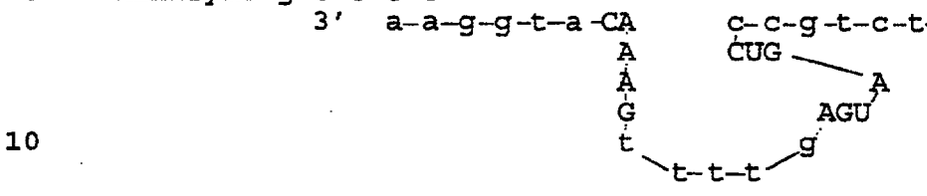
Minizyme t t t t



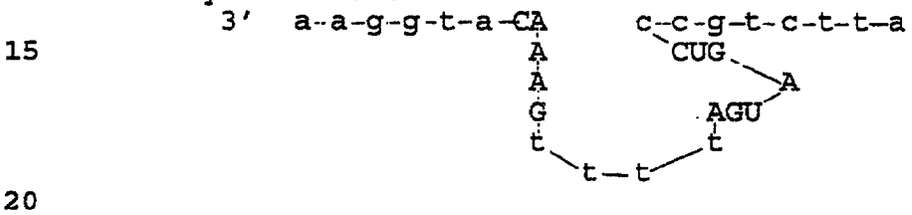
CAT system. (Seq ID No.14-17)

Substrate (17-mer) 5' UUCCAUGUC GGCAGAAt 3'

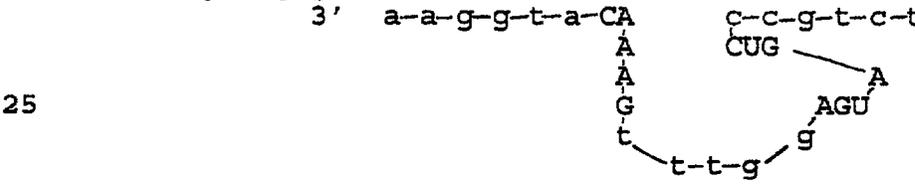
Minizyme g t t t t



Minizyme t t t t



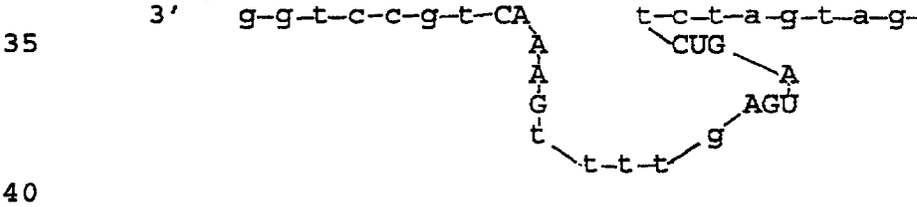
Minizyme g g t t t



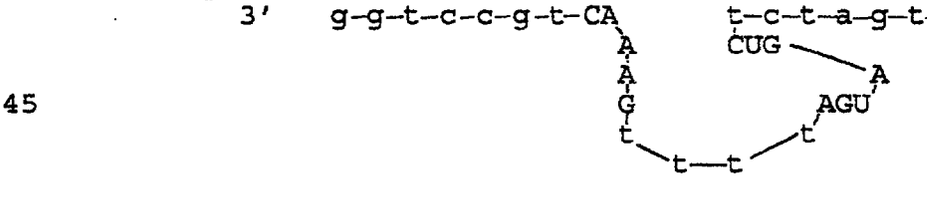
Tumor Necrosis Factor α(TNFα) system. (Seq ID No. 18-20)

Substrate (20-mer) 5' CCAGGCAGUC AGAUCAUCUt 3'

Minizyme g t t t t



Minizyme t t t t



3. Observed cleavage rate (k_{obs} min^{-1}) and extent of cleavage (%P) of short substrates by minizymes with various linkers. ("Table 1B)".

Experimental conditions: 10mM $MgCl_2$, 50 mM Tris.HCl, pH 8.2 (for Interleukin-2 and TAT systems) and pH 8.0 (for CAT and TNFα systems), 37°C, [Substrate]= 2μM, [Minizyme]= 5μM (except [TNCMtttt, TNFMgtttt] = 4.3μM).

	Expt.1		Expt.2		Expt.3		Mean			
Minizyme linker	k_{obs}	%P.	k_{obs}	%P.	k_{obs}	%P.	k_{obs}	(ó)	%P.	(ó)
Interleukin-2										
5'x3'										
cctt	0.010	80#	0.011	80#			0.011	(.001)	80#	
tttt			0.052	81.1	0.049	84.2	0.051	(.002)	83	(2)
ttttt			0.058	86.1	0.064	82.1	0.061	(.004)	84	(3)
ttttc	0.016	76.3	0.015	80.0			0.016	(.001)	78	(3)
gttt	0.118	82.3	0.107	84.5			0.113	(.008)	83	(2)
gtttt	0.299	83.5	0.287	85.3	0.225	84.9	0.270	(.040)	85	(1)
gttttt	0.287	82.4	0.253	84.1			0.270	(.024)	83	(1)
gttttt	0.200	84.3	0.169	88.6			0.185	(.022)	86	(3)
gttta	0.026	85.9	0.022	94.7			0.024	(.003)	90	(6)
gtttta 0.047	81.0	0.038	88.4				0.043	(.006)	85	(5)
gtttg	0.122	82.3	0.092	89.8	0.107	83.9	0.107	(.015)	85	(4)
gttttg	0.101	82.1	0.094	84.2			0.098	(.005)	83	(2)
ggttt 0.049	82.4	0.048	89.4				0.049	(.001)	86	(5)
gtgtt	0.178	83.0	0.181	83.2			0.180	(.002)	83	(1)
gttgt	0.065	93.9	0.072	85.3			0.069	(.005)	90	(6)
gttt ($dg^{12}dc^{15.2}$)	0.041	90.0	0.040	91.7			0.041	(.001)	91	(1)

Minizyme linker	Expt.1 k_{obs}	Expt.1 %P.	Expt.2 k_{obs}	Expt.2 %P.	Expt.3 k_{obs}	Expt.3 %P.	Mean k_{obs} (ó)	Mean %P. (ó)
TAT								
tttt	0.074	89.1	0.067	91.9			0.071 (.005)	91 (2)
gtttt	0.179	89.7	0.190	90.8			0.185 (.008)	90 (1)
CAT								
tttt	0.186	82.9	0.175	84.7			0.181 (.008)	84 (1)
gtttt^	0.526* 60.8		0.448*	63.8	0.478* 62.8		0.48* (0.04)	62 (2)
gtttt^	0.791* 72.0		0.809*	71.4			0.80* (0.01)	72 (1)
gtttt^	0.650* 63.0							
gtttt^	0.453* 61.0							
gtttt^^							0.59* (0.16)	65 (5)
ggttt	0.278*	68.8	0.352*	67.6			0.32* (0.05)	68 (1)
TNFα								
tttt	0.002	70.0#	0.002	70.0#			0.002 (.001)	70.0#
gtttt	0.274	59.0	0.180	74.2	0.303	58.7	0.25 (0.06)	64 (9)

fixed at this value.

^ four different syntheses, ^^ average of data for the four syntheses.

* reaction is biphasic; rate constant for the initial faster reaction is given.

4. Minizymes with 5'd(GTTTT) linkers have improved cleavage activity *in vitro* compared with those with 5'd(TTTT) linkers.

Table 2

System	k_{obs} (Mgtttt)	k_{obs} (Mtttt)	k_{obs} (Mgtttt)/k_{obs}(Mtttt)
Interleukin-2	0.270 0.051		5.3
TAT	0.185 0.071		2.6
CAT	0.59	0.181	3.3
TNFα	0.25	0.002	125

The data in table 2 show that the minizymes with gtttt linkers consistently show k_{obs} values of 0.2 min^{-1} or better. Since we know that IL2Mgtttt, with k_{obs} = 0.27 min^{-1}, is active in cells (see Example 3), we can conclude that a minizyme with at least this level of activity in an in vitro system should not be hindered in cells by its k_{obs} value (i.e. rate of cleavage should not be rate limiting in cells), all other things being equal (such as target site being accessible).

Example 2. Minizyme Suppression of CAT Expression in CHO Cells.

Introduction

Minizymes are sequence specific RNA endonucleases derived from standard hammerhead ribozymes by elimination of helix II. Minizymes have been shown to exhibit significant in vitro cleavage activity against both short RNA targets as well as long transcribed RNA. This report describes the testing of a particular minizyme targeted against the mRNA of CAT (Chloramphenicol acetyl transferase) expressed in a mammalian cell line. The minizyme is a chimeric DNA/RNA molecule synthesized by solid phase methods and transfected into a CHO (chinese hamster ovary) cell line stably expressing CAT.

Experimental Protocol

A CHO based CAT expressing cell line MC 11, in which CAT is expressed from the Human metallothionein IIA (MT) promoter, was used in all experiments. The MT promoter is transcriptionally active at very low metal concentration and a reasonable level of CAT expression is observed in the absence of induction by added metals.

8×10^4 cells were plated out in EMEM containing 10% foetal calf serum and allowed to attach overnight (14-16 hours). The cells were washed once with 1 x PBS to remove serum, then the test molecules (pre-treated for 30 minutes with 1μL of lipofectamine (GIBCO BRL, Life Technologies, Maryland, USA) in serum-free EMEM) were transferred to the cells. The final concentration of test molecules was 10 μM. After four hours both serum and Alamar Blue (Alamar Bio-Sciences Inc, Sacramento CA) (each to a final concentration of 10%) was added to the cells and incubation continued for a further 18 hours. At this time the supernatant was removed and the cumulative cell metabolic activity determined by measuring the extent of reduction of the Alamar Blue reagent. The cells were harvested and CAT activity assayed (Sleigh, 1986).

Alamar Blue Assay.

Alamar Blue is a commercial material designed for use in cytotoxicity assays for cells in culture. The reagent is reduced intracellularly in an energy dependent fashion. The reduced form of the reagent is readily quantified by either its absorption spectrum or by fluorescence. We have quantified the reduced form of Alamar Blue by absorption spectroscopy.

Target mRNA

The target site in the CAT mRNA corresponds to CAT site 3 described in Haseloff and Gerlach (1988). The cleavage triplet is a GUC site and is located towards the 3' end 662 nucleotides from the ATG start codon.

Sequences of molecules

The test molecules are as follows (Seq ID No. 21-24):
Lower case letters are DNA, upper case letters are RNA.

tRNA	Yeast tRNA (Sigma) Phenol/Chloroform extracted.
N_{17}	5' nnn nnn nnn nnn nnn nn 3' (n = a, g, c or t)
CAT Antisense	5' att ctg ccg aca tgg aa 3'
CAT Minizyme	5' att ctg cc CUGAUGA gtttt GAAAC atg gaa 3'
CAT Inactive Minizyme	5' att ctg cc CUGAUGA gtttt GAGAC atg gaa 3'

Bold G in CAT Inactive Minizyme represents the mutation that inactivates the minizyme.

Results.

Table 3.

Data from three Independent Experiments

Treatment	Replicas	CAT Activity ± SEM	Alamar Blue ± SEM
		Experiment 1'	
serum starved + lipofectamine	3	9385 ± 318	1.08 ± 0.06
Minizyme	3	3788 ± 603	1.19 ± 0.06
Antisense	3	3885 ± 490	0.975 ± 0.05
Inactive Minizyme	3	7043 ± 695	1.085 ± 0.01 (n = 2)
N_{17}	3	7254 ± 876	0.966 ± 0.06
		Experiment 2'	
serum starved + lipofectamine	2	10573 ± 455	1.045 ± 0.03
tRNA	3	9680 ± 656	0.938 ± 0.04
Minizyme	3	4943 ± 974	0.953 ± 0.02
Antisense	3	5073 ± 467	0.883 ± 0.01
Inactive Minizyme	3	6641 ± 642	0.872 ± 0.05
N_{17}	3	4260 ± 342	0.857 ± 0.05
		Experiment 3'	
serum starved + lipofectamine	2	4143 ± 269	0.89 ± 0.05
tRNA	3	5646 ± 235	0.781 ± 0.02
Minizyme	3	2855 ± 491	1.031 ± 0.07
Antisense	3	2978 ± 259	0.712 ± 0.10
Inactive Minizyme	3	5969 ± 225	0.778 ± 0.04
N_{17}	3	2210 ± 195	0.767 ± 0.04

Table 4

Mean ± SEM CAT Expression as a percentage of Control.
(Controls are serum starved + lipofectamine treated cells)

Treatment	Experiment 1	Experiment 2	Experiment 3	Mean ± SEM
Control	100 ± 3.3	100 ± 4.3	100 ± 6.5	**100**
tRNA	-	91.6 ± 6.8	136.3 ± 4.2	**114 ± 22**
Minizyme	40.4 ± 15.9	46.8 ± 19.7	68.9 ± 17.2	**52 ± 9**
Antisense	41.4 ± 12.6	48.0 ± 9.2	71.9 ± 8.7	**54 ± 9**
Inactive Minizyme	75.0 ± 9.9	62.8 ± 9.7	144.1 ± 3.8	**94 ± 25**
N_{17}	77.3 ± 12.1	40.3 ± 8.0	53.3 ± 8.8	**57 ± 11**

Figure 1 shows the CAT Expression (Mean ± SEM) for Different treatments. Each experiment is performed in triplicate. Figure 2 shows the extent of Alamar Blue Reduction (mean ± SEM) in the same experiments as in Figure 1. Extent of reduction relates to metabolic activity of the cells during the 18 hours post-transfection.

Discussion.

The CAT minizyme contained the new linker 5'gtttt. When tested against a short 17-mer synthetic RNA substrate in vitro at 37°C, the minizyme cleaved the substrate with a reasonable rate constant (~ 0.5 min^{-1}, $t^1/_2$ ~ 1.4 minutes). In all experiments a constant number of cells (8 x 10^4) were seeded and treated identically with the exception of the added oligonucleotides. Based on observed CAT activity, the random 17-mer does not appear to be an appropriate control molecule. Accordingly tRNA was included in two of the experiments to provide an alternative control.

The minizyme and the DNA antisense show similar levels of suppression (52 ± 9%, 54 ± 9%, respectively) and both are significantly more effective than the inactivated minizyme (94 ±

25%, mean results Table 4). Thus both the minizyme and the antisense are showing activity in this cultured cell system.

We have been mindful of the danger of selecting a single protein or mRNA level as a specificity control and have therefore monitored the rate of general metabolism (by Alamar Blue reduction) as a measure of the specificity of the test molecules. It is interesting to note that the minizyme is apparently less toxic than the antisense in all experiments (Table 3, Figure 2). Therefore the relative activity of the minizyme may be greater than that of the antisense, since a proportion of the apparent activity of the antisense molecule could result from a reduction in cell metabolism compared with minizyme treated cells.

Example 3

The activities of DNA-armed ribozymes and minizymes against Interleukin-2 mRNA in vivo.

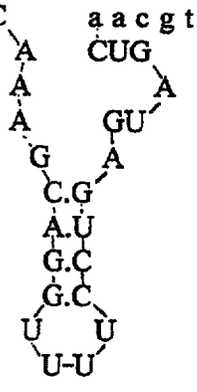
The molecules tested.

The following molecules (2-7) have been synthesized and tested for activity using one or more of the assays described below. Molecule 2 is a hammerhead ribozyme with deoxyribonucleotides in the arms which hybridize to the substrate. Molecule 3 is a minimized hammerhead ribozyme (minizyme) with a linker of sequence d(GTTTT) replacing stem-loop II of the full-sized ribozyme. Molecule 4 is a minizyme with 5′ d(GTTTT) linker, which has been rendered inactive by replacing the conserved A_{15} by a G. Molecule 5 is a DNA antisense control with sequence complementary to the 15-nucleotide target sequence. Molecule 6 is a DNA control with the same base composition as the DNA antisense, but with scrambled sequence. Molecule 1 is a 15-nucleotide, synthetic substrate with the same sequence as that targeted in the IL2 m-RNA; it is used to determine cleavage rate constants for the ribozyme and minizymes <u>in vitro</u>. In the sequences below, ribonucleotides are denoted by

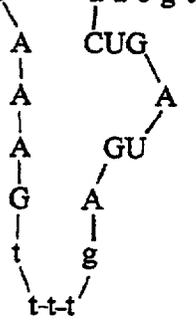
upper-case letters and deoxyribonucleotides by lower-case letters (Seq ID No. 25-31).

1. 5' UCCUGUCUUGCAUUG — interleukin-2 target sequence IL2S15

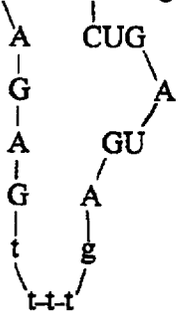
2. 3' a g g a C A A A G G A C.G A.U G.C G.C U U U-U a a c g t a a c 5' — DNA-armed ribozyme IL2R-DNA



3. 3' a g g a C A A A G G t t-t-t a a c g t a a c 5' — minizyme with dGTTTT linker $IL2M_{GTTTT}$



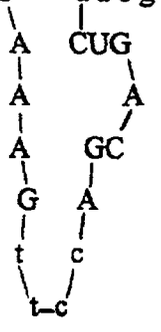
4. 3' a g g a C A G A G G t t-t-t a a c g t a a c 5' — inactive minizyme (GAAAC - GAGAC) $IL2M_{Inactive}$



5. 3' aggacagaacgtaac 5' DNA antisense control IL2AS

6. 3' gaatcgcagaaagca 5' DNA random-sequence control IL2C

7. 3' aggaC aacgtaac 5' minizyme with dCCTT linker $IL2M_{CCTT}$



Preparation of oligonucleotides

The oligonucleotides were synthesised on an Applied Biosystems (Foster City, CA) model 391 synthesiser using protected DNA phosphoramidite monomers and RNA monomers, protected at the 2'-hydroxyl by tert-butyldimethylsilyl groups, from Millipore (Marlborough, MA). For convenience in the syntheses, the 3' nucleotide in all molecules is a deoxyribonucleotide. The methods used for the deprotection and gel-purification of the oligonucleotides were as described previously (McCall et al., 1992), with the exception that the oligonucleotides used for testing in cells were precipitated twice (rather than once) from 0.3 M sodium acetate and 80% ethanol, and then washed twice (rather than once) in cold 80% ethanol before drying under vacuum. The oligonucleotides were re-dissolved in autoclaved, de-ionized water. The concentrations of the oligonucleotide solutions were determined by UV spectroscopy using the following molar extinction coefficients for the

nucleotides at 260 nm: A 15,400, G 11,700, C 7,300, T and U 8, 800 $Lmol^{-1}cm^{-1}$. The purity of each oligonucleotide was checked by phosphorylating the 5′ end using [γ-^{32}]ATP and polynucleotide kinase (Boehringer Mannheim, Germany) as described previously (McCall et al., 1992). The oligonucleotides were stored at -20°C.

Determining rates of cleavage (k_{obs}) by minizymes

The rates at which the minizymes ($IL2M_{2'5'}$ and $IL2M_{CC'T}$) and ribozyme (IL2R-DNA) cleaved their cognate, short substrate (IL2S15) were determined in 10 mM $MgCl_2$, 50 mM Tris.HCl buffer, pH 8.25, at 37°C with 5 μM minizyme and 2 μM substrate. The substrate was labelled on its 5′ end with [^{32}P]-phosphate. The minizyme and substrate were heated together in buffer for two minutes at 70°C, put to 37°C for 2 minutes, centrifuged briefly at room temperature, and then returned to 37°C for 2 minutes before starting the reaction by adding $MgCl_2$ (at 37°C) to the mixture. The volume of the reaction mixture was generally 30 μL. 2 μL samples were taken at various times, and the reactions in these were quenched by adding 4 μL of 20 mM EDTA, 80% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol. The samples were electrophoresed on a 15% polyacrylamide gel containing 7M urea, and the amounts of 5′-product and uncleaved substrate were quantified using a Molecular Dynamics PhosphorImager (Sunnyvale, CA) and ImageQuant software. Kinetic parameters were obtained by fitting the data for % of product formed (P_t) versus time (t) to the equation

$$P_t = P_\infty - (\exp(-k_{obs}t)P_\Delta)$$

where P_t is the amount of product at time t, P_∞ is the amount of product at $t = \infty$, k_{obs} is the first-order rate-constant for the reaction, and P_Δ is the difference between the percentage of product at $t = \infty$ and $t = 0$. This is a conventional first-order kinetic equation from which k_{obs}, P_∞ and P_Δ are determined by least-squares fitting of the data. The quoted rate constants

are the mean (± standard deviation) of at least two experiments.

Assays for interleukin-2.

Abbreviations: PBMN (peripheral blood mononuclear cells), PHA (phytohemagglutinin), IL-2 (interleukin-2).

Interleukin-2 is synthesised and secreted by T-lymphocytes following their activation by antigens or mitogens. In experiments to determine how well the test molecules could suppress the production of IL-2, an enriched population of human PBMN T-cells were transfected with the test molecules, and then stimulated with PHA to produce IL-2. In all cases, these test molecules were co-transfected with 1 μg tRNA and their effects on IL-2 levels were determined relative to the effect of the control which was 1μg tRNA. The amounts of IL-2 secreted were assayed by three methods. In one method, IL-2 was assayed indirectly by measuring the ability of the supernatant from the PBMN cells to promote the growth of IL2-dependent mouse CTLL cells. In another, since IL-2 produced by PBMN T-cells promotes the growth of the same cells, IL-2 levels were assayed directly by measuring the growth of the stimulated cells. In addition to these bio-assays, immunoassays (which measure free and receptor-bound IL-2) were performed.

Indirect bio-assay.

This assay is specific for interleukin-2 and is based on the absolute requirement of IL-2 for the growth of CTLL cells. Before use, the CTLL cells, maintained continuously proliferating in complete media containing added IL-2, were washed 3 times in complete media without added IL-2(Tables 13a and 13b).

In microtitre trays 100,000 PBMN cells (enriched in T-cells) were transfected in complete medium (RPMI plus 10% foetal-calf serum) with the test and control molecules at 5, 10 and 20 μM using 25 μg/mL DOTAP (Boehringer Mannheim, Germany) according to the manufacturer's instructions. After a period of 7 hours, the cells were stimulated with 5 μg/mL PHA (Sigma). After an additional 15-20 hours, supernatants from the PBMN cells were serially diluted and, in triplicate, 100 μL from each dilution were added to 5000 CTLL cells in 20 μL media. The CTLL cells were allowed to grow for 20 hours, were pulsed with ^{3}H-thymidine for 4 hours, were harvested, and then the DNA-associated radioactivity was determined. Slow growth of the CTLL cells (low level of incorporated ^{3}H-thymidine) indicates low levels of interleukin-2 in the supernatant, and hence a test molecule with good activity against interleukin-2 in the PBMN cells. The percentage inhibition of IL-2 by the test molecules is measured in the range where the cell growth-rate is linearly dependent on the amount of IL-2 (Sioud, 1994).

Direct bio-assay.

PBMN cells were transfected with the test molecules for 12 hours, stimulated with PHA for about 48 hours, pulsed with ^{3}H-thymidine, and then harvested 18 hours later to determine DNA-associated radioactivity (Tables 14a and 14b).

Enzyme Amplified Sensitivity Immuno-Assay (EASIA).

Total IL-2 in the supernatant from PBMN cells transfected with 20μM IL2M$_{\text{stem}}$ and 20μM IL2M$_{\text{inactive}}$ was determined using IL2-EASIA, a solid-phase Enzyme Amplified Sensitivity Immuno-Assay performed in microtitre plates according to the manufacturer's instructions (MADGENIX) (Table 15).

Toxicity to cells.

The viability of PBMN cells which had been transfected with test and control molecules was determined using acridine orange and ethidium bromide. Dead cells appear orange and live cells appear green in the presence of these indicators. The assays were performed on PBMN cells that were transfected with the test and control molecules for a period of 22-27 hours; these cells were not stimulated with PHA since this results in cell aggregation and hence inaccuracy in cell counting. In parallel, cells from the same donor were transfected with the test and control molecules and stimulated with PHA, and when supernatants from these cells were collected for assaying IL-2 levels, the viabilities of the cells not stimulated by PHA were determined.

Activities of DNA-armed ribozymes and minizymes.

The original set of molecules synthesized for testing contained the DNA-armed ribozyme, the minizyme with d(CCTT) linker, the DNA antisense, and the DNA random-sequence control (molecules 2, 7, 5 and 6 respectively). In these first experiments using the indirect assay, with transfection concentrations of test molecules at 10μM, the DNA-armed ribozyme showed good activity against IL2, while the minizyme with d(CCTT) linker and the DNA random-sequence control showed no activity; the DNA antisense showed about 50% the activity of the DNA-armed ribozyme. The activities of the DNA-armed ribozyme and the minizyme in cells correlated with the observed cleavage activities of these molecules as measured against the short synthetic IL2 substrate in vitro. Since the cleavage rate shown by the minizyme with d(CCTT) linker was extremely slow, we investigated the reason for this poor activity. In this investigation we found that by changing the linker from 5' d(CCTT) to 5' d(GTTTT) the cleavage rate of the minizyme could be increased 25-fold. The observed cleavage rate constants in 10 mM $MgCl_2$, at pH 8.25 and 37°C, for the DNA-armed ribozyme, the minizyme with d(CCTT) linker, and the new minizyme with d(GTTTT) linker, against their

cognate, short substrate are 1.4 (0.2), 0.011 (0.001) and 0.27 (0.04) min^{-1}, respectively.

Following the discovery of the highly active minizyme with d(GTTTT) linker, we synthesized in large scale the DNA-armed ribozyme, the minizyme with d(GTTTT) linker, an inactivated minizyme with d(GTTTT) linker, the DNA antisense, and the DNA random-sequence control (molecules 2-6 above), and tested them for activity in cells.

Results from inpedendent experiments using molecules 2-6.

In the tables below, the effectiveness of each test molecule (at different concentrations) in inhibiting the production of IL2 is presented as % inhibition averaged over two or three independent experiments (each done in duplicate or triplicate) with standard deviations given in brackets. In the first of each pair of tables, the data show the effectiveness of the test molecules against IL2, relative to that of the DNA random-sequence control taken as having 0% inhibitory effect. Since ribozymes and minizymes are being developed as alternatives to other oligonucleotide-based therapies, we believe that the inhibitory effects of these molecules, over and above the non-specific effects of a randomly-chosen oligonucleotide, are the data of interest. In most experiments, tRNA was also included as an additional control molecule. Generally, the DNA random-sequence control showed some activity against IL2 relative to tRNA, and so these data are presented for information (relative to tRNA taken as having 0% inhibitory effect) in the second of each pair of tables.

Indirect assay. **% inhibition of IL2 as measured by the indirect assay.**

Table 13A. Data relative to DNA random-sequence control.

Test molecule	Transfection concentration 5 μM	10μM	20μM
IL2R-DNA	33	42 (13)	69 (1)
$IL2M_{GTTTT}$	31	41 (4)	53 (11)
$IL2M_{Inactive}$	5	6 (27)	28 (6)
IL2AS	10	21 (19)	13 (6)

Table 13B. Data relative to tRNA.

Test molecule	Transfection concentration 5 μM	10μM	20μM
IL2R-DNA	45	51 (9)	80 (4)
$IL2M_{GTTTT}$	43	51 (3)	73 (12)
$IL2M_{Inactive}$	22	22 (17)	47 (12)
IL2AS	25	35 (9)	49 (15)
IL2C	18	16 (7)	35 (9)

Direct assay. **% inhibition of IL2 as measured in individual experiments by the direct assay.**

Table 14A. Data relative to DNA random-sequence control.

Test molecule	Transfection concentration 10 μM
IL2R-DNA	67 (3)
$IL2M_{GTTTT}$	46 (6)
$IL2M_{Inactive}$	24 (2)
IL2AS	28 (14)

Table 14B. Data relative to tRNA.

Test molecule	Transfection concentration 10 μM
IL2R-DNA	73 (4)
$IL2M_{GTTTT}$	55 (7)
$IL2M_{INactive}$	37 (2)
IL2AS	40 (14)
IL2C	18 (4)

EASIA

Table 15. Transfection concentration 20 μM.

Test molecule	IU/ml	% inhibition relative to tRNA
$IL2M_{GTTTT}$	70	72 %
$IL2M_{Inactive}$	120	52 %
control (tRNA)	250	-

Summary of results.

The DNA-armed ribozyme (IL2R-DNA) and the minizyme with d(GTTTT) linker ($IL2M_{GTTTT}$) show activity specifically against interleukin-2 in human PBMN cells. The effects of these two molecules are greater than those measured for a DNA antisense molecule (IL2AS) and an inactivated minizyme ($IL2M_{Inactive}$) directed to the same target site on the IL2 mRNA, at transfection concentrations of 5, 10 and 20 μM. The molecules do not appear to be toxic to cells over a 24-hour period.

Example 4
Mini-ribozymes

Comments in publications on hammerhead ribozymes teach that helix II can be reduced to 2 b.p. without loss of activity, but further reduction to 1 b.p. results in at least a 10-fold reduction in activity (see data from Tuschi and Eckstein (1993) and from Long and Uhlenbeck (1994)). Note that these data are for all-RNA ribozymes. We have found that ribozymes with 1 b.p. in helix II, such that the sequence of "stem-loop II" is 5'GTTTC or 5'GTTTC, where T may be dT or rU, have better than 10% the activity of analogous ribozymes with 4 b.p. in helix II. We call such ribozymes "mini-ribozymes". In some cases, the mini-ribozymes have observed cleavage constants which exceed that of the full-sized analogous ribozymes. Based upon published data, these findings are totally unexpected. Our data are presented below.

Sequences of mini-ribozymes and ribozymes.

Upper-case letters represent ribonucleotides, and lower-case letters represent deoxyribonucleotides. Substrates are labelled S, followed by the number of nucleotides in the substrate molecule. Minizymes are labelled M, followed by the sequence of the linker connecting the conserved nucleotides A_9

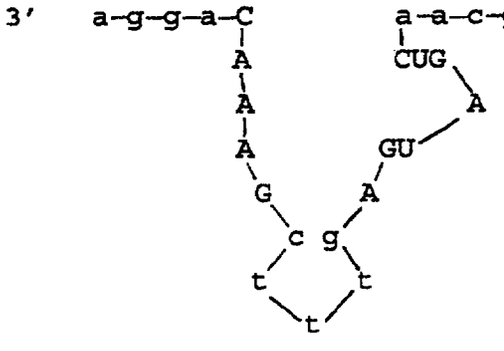
and G_{12} (e.g. KrMgtttc is a miniribozyme in the Kruppel system, with a deoxyribonucleotide linker of sequence 5'd(GTTTTC). Full-sized ribozymes which contain a stem-loop II (with 4 b.p. in helix II) are labeled RA (if made of ribonucleotides), RB (with deoxyribonuclotides in the arms which hybridize to the substrates, and ribonucleotides elsewhere), and RC (if made of deoxyribonuclotides, except for the conserved ribonucleotides C_3-A_9 and C_{12}-$C_{15.2}$).

Interleukin-2 system (Seq ID No. 32-38)

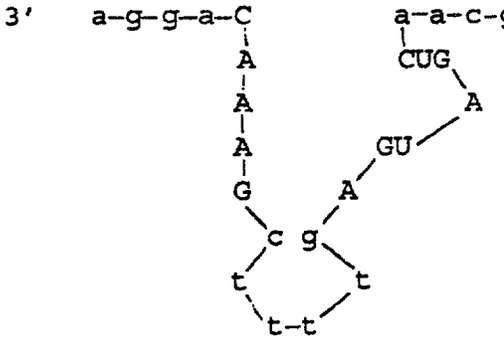
IL2S15 (15 mer substrate)

5' UCCUGUCUUGCAUUg 3'

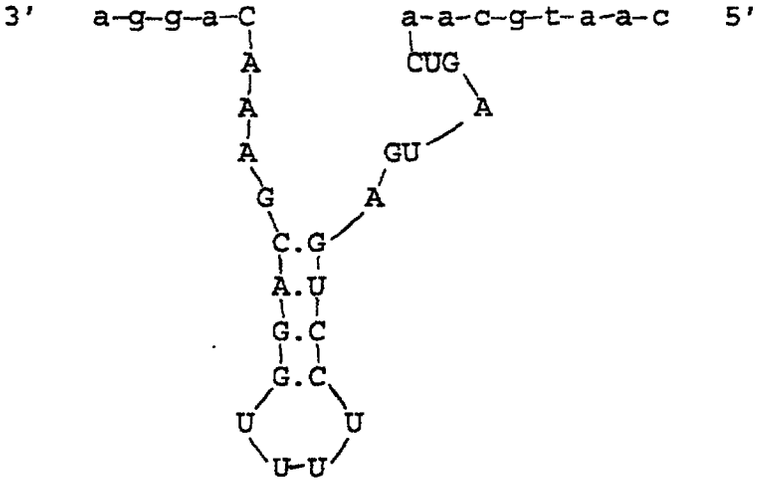
IL2Mgtttc (DNA/RNA mini-ribozyme with d(GTTTC) linker



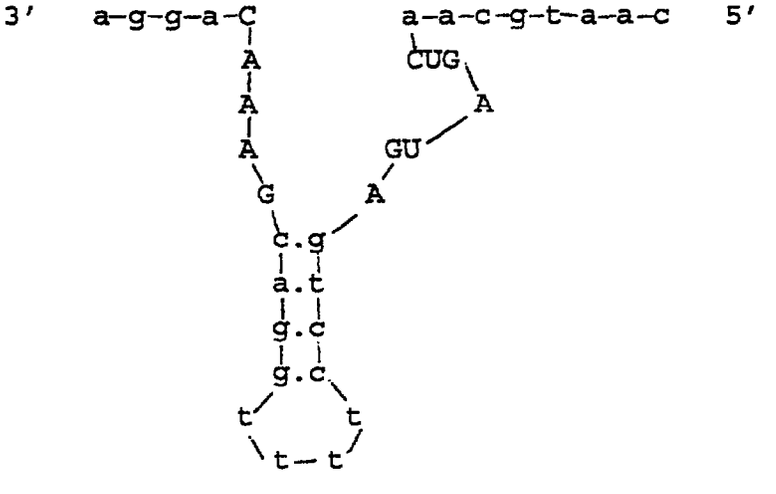
IL2Mgttttc (DNA/RNA mini-ribozyme with d(GTTTTC) linker



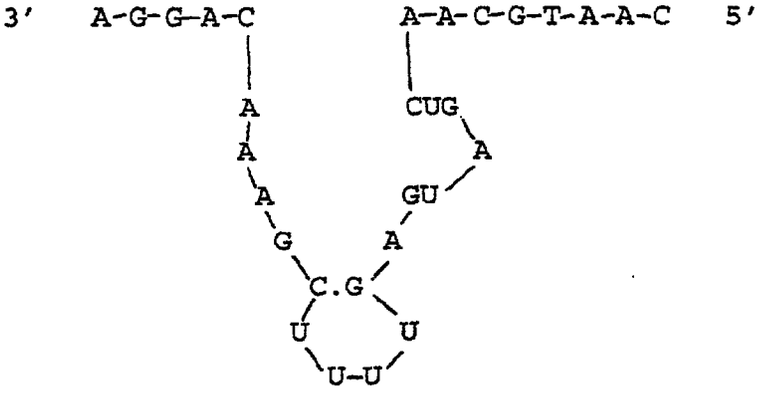
IL2"RB" DNA-armed ribozyme



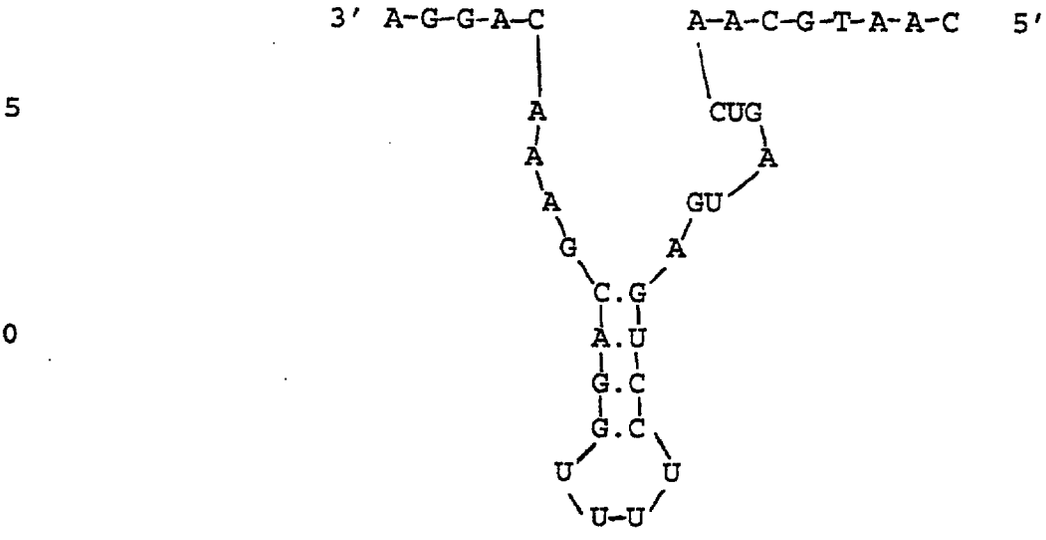
IL2"RC" DNA-containing ribozyme



IL2 MGUUUUC(all-RNA miniribozyme with r(GUUUUC) linkers



IL2 (all RNA ribozyme)



TAT system (Seq ID No. 39-43)

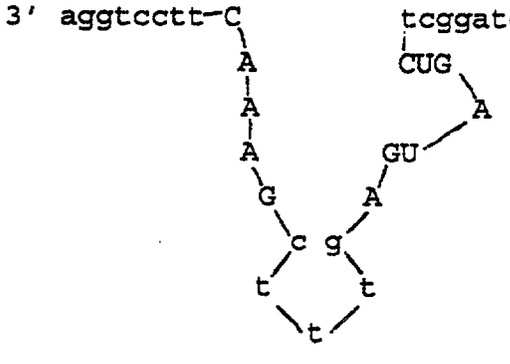
TATS13 (13 mer substrate)

5' GGAAGUCAGCCU a 3'

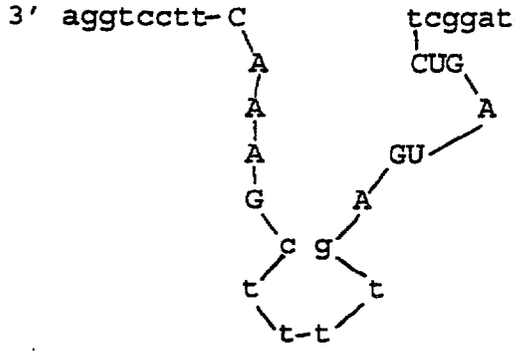
TATS21 (21 mer substrate)

5' UCCAGGAAGUCAGCCUAGGA c3'

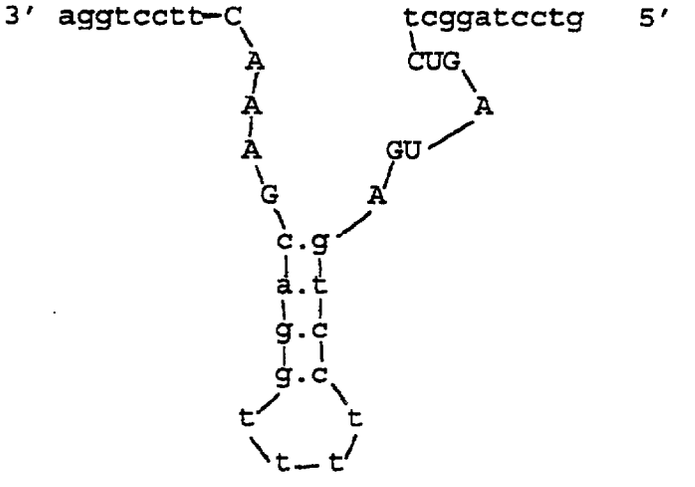
TATMgtttc (DNA/RNA mini-ribozyme with d(GTTTC) linker



TATMgttttc (DNA/RNA mini-ribozyme with d(GTTTTC) linker



TAT"RC" DNA-containing ribozyme



Kr system (Seq ID No. 44-47)

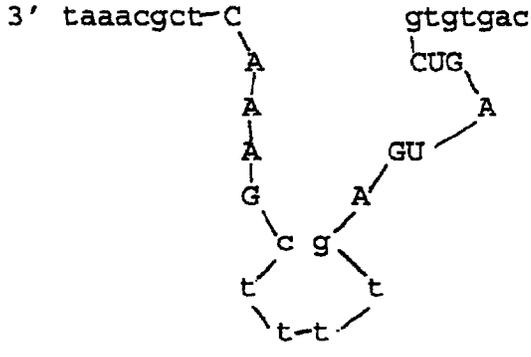
KrS13 (13 mer substrate)

5' GCGAGUCCACAC T 3'

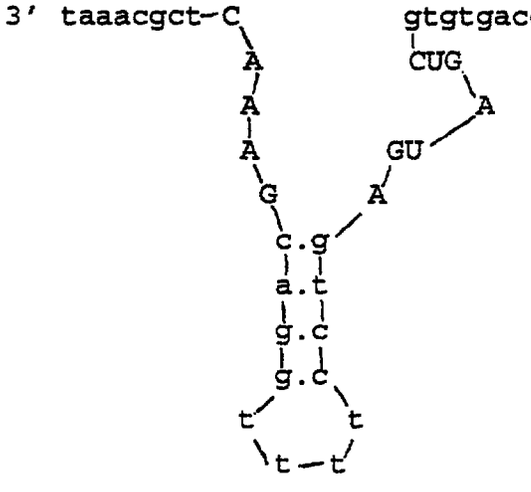
KrS21 (21 mer substrate)

5' AUUUGCGAGUCCACACACUGGA g 3'

KrMgttttc (DNA/RNA mini-ribozyme with d(GTTTTC) linker



Kr "RC" DNA-containing ribozyme



Experimental data.

The method for determining rates of cleavage (kobs) of short substrates by the miniribozymes is as described in Example 1.

Initial experiments at pH 8.2 showed the reactions for the TAT and IL2 miniribozymes and "RC" ribozymes are very fast, with the reactions being completed in less than 1 minute. Therefore, the reactions were performed at the lower pH of 7.1 (which in principle should be 12.6-fold slower than the pH 8.2 if the reactions have a first order dependence on OH concentration in this pH range). The data at pH 7.1 show more accurately the relative activities of the various molecules. In the Tables below, values in parentheses(σ) are the standard deviations of at least two independent determinations; and *indicates an initial fast reaction followed by a slower reaction, with for the initial fast reaction being given.

Table 8. k_{obs} (min-1) and %P∞ for KrMgttttc and KrRC cleaving the 13-mer and 21-mer substrates in 10 mM $MgCl_2$, 50 mM Tris.Cl pH 7.1, 37°C, 6μM ribozyme/miniribozyme and 4μM substrate.

	KrMgtttc		KrRC	
	$k_{obs}(\sigma)$	$\%P_\infty\sigma$	$k_{obs}(\sigma)$	$\%P_\infty\sigma$
KrS13	0.59* (0.09)	70(3)	1.6* (0.3)	78(2)
KrS21	3.0* (0.2)	58(1)	1.34* (0.08)	56(1)

Table 9. k_{obs} (min^{-1}) and $\%P_\infty$ for TATMgttte, TATMgtttte and TATRC cleaving various substrates in 10mM $MgCl_2$, 50 mM Tris.Cl pH 7.1, 37°C. Concentration of ribozyme exceeded that of substrate, with concentrations varying between 4μM and 8μM (ribozyme and miniribozyme) and 2μM and 5μM (substrate), with typical concentrations being 6μM for ribozyme/miniribozyme and 4μM for substrate.

	TATMgtttc		TATMgttttc		TATRC	
	$k_{obs}(\sigma)$	$\%P_\infty\sigma$	$k_{obs}(\sigma)$	$\%P_\infty\sigma$	$k_{obs}(\sigma)$	$\%P_\infty\sigma$
TATS13	0.06(0.02)	78(6)	0.175* (0.003)	74(7)	0.43* (0.008)	80 (7)
TATS21	1.1*(0.3)	39 (1)	0.9* (0.2)	45 (3)	0.45* (0.09)	31 (2)

Table 10. k_{obs} (min^{-1}) and $\%P_\infty$ for the DNA-containing IL2Mgtttt, IL2Mgttttc, IL2Mgtttc, II2RB and IL2RC, and the all-RNA IL2MGUUUUC and IL2RA cleaving the substrate IL2S15-6/8 in 10 mM $MgCl_2$, 50 mM Tris. Cl pH 7.1, 37°C. Concentration of ribozyme exceeded that of substrate, with concentrations varying between 2.5 μM and 20 μM (ribozyme), and 1μM and 8μM (substrate, with typical concentrations being 5 μM for ribozyme/miniribozyme and 2 μM for substrate.

	$k_{obs}(\sigma)$	$\%P_\infty\sigma$
IL2Mgtte	0.119 (0.0004)	85(4)
IL2Mgtttte	0.19 (0.02)	82(1)
IL2RB	0.06 (0.02)	78(5)
IL2RC	0.05 (0.001)	90(7)
IL2MGUUUUC	0.316 (0.0001)	76(2)
IL2RA	1.4* (0.2)	66(1)

Table 11. Comparing the rates of cleavage of substrates (at 37°C, pH 7.1, 10 mM $MgCl_2$) by the miniribozymes with d(GTTTC) and d(GTTTTC) linkers relative to that analogous DNA-containing ribozymes RC, and the all-RNA mini-ribozyme with r(GUUUUC) linker relative to the analogous all RNA ribozyme RA.

	k_{obs} gtttc/RC	k_{obs} gttttc/RC	k_{obs} GUUUUC/RA
KrS13	-	0.37	-
KrS21	-	2.2	-
TATS13	0.14	0.41	-
TATS21	2.4	2.0	-
IL2S15	2.4	3.8	0.23

Comments.

Several of the molecules used (Kr"RC", TAT"RC", IL2S15, IL2Mgttte and IL2"RC") have been synthesized at least twice. The rates of cleavage and extents of cleavage do not vary significantly for molecules from different syntheses.

The IL2 minizyme with d(GTTTT) linker, which has kobs = 0.27 min^{-1} at pH 8.2 (and kobs = 0.024 (0.005) min^{-1} at pH 7.1), is active in cells (see Example 3). Therefore, the IL2 mini-ribozymes with d(GTTTC) and d(GTTTTC) linkers, which have kobs = 0.11 and 0.19 min^{-1}, respectively, at pH 7.1, and which target the same site in the IL2 mRNA, are expected to be active in cells. For similar reasons, the all-RNA IL2 mini-ribozyme IL2MGUUUUC, which has kobs = 0.316 min at pH 7.1, is expected to be active in cells.

Example 5 In preliminary experiments, a miniribozyme targeted against interleukin-2 mRNA was shown to have activity against this target in human peripheral blood mononuclear cells. The sequence of the miniribozyme, IL2MGUUUUC, was 5′ CAAUGCAA CUGAUGA GUUUUC GAAAC AGGa 3′ (SEQ. ID NO. 48) where upper-case letters represent RNA and lower-case letters DNA (the 3′ nucleotide was DNA for convenience in the chemical synthesis). The experiment to test for activity in cells was performed in the manner described in Example 3 (pages 47-57).

Example 6 The interleukin-2 miniribozyme cleaves an 809-nucleotide interleukin-2 transcript much faster in vitro at 37°C than does an interleukin-2 ribozyme; time taken for 50% of the IL2 transcript to be cleaved is 30 minutes for the miniribozyme and 4.6 hours for the ribozyme. Also, the miniribozyme cleaves the interleukin-2 transcript faster than does the ribozyme over a wide temperature range (please see Figure 5).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Commonwealth Scientific and Industrial Research Organisation

(ii) TITLE OF INVENTION: OPTIMIZED MINIZYMES AND MINIROBOZYMES AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 50

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Davies Collison Cave
(B) STREET: 1 Little Collins Street
(C) CITY: Melbourne
(D) STATE: Victoria
(E) COUNTRY: Australia
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version # 1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/488181
(B) FILING DATE: 07-JUN-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/574396
(B) FILING DATE: 18-DEC-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Slattery, John M.
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +613 9254 2777
(B) TELEFAX: +613 9254 2770

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

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

GCGGGUCAUG AAG 13

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

GACACUUCAU CUGAUGAGUC CUUUUGGACG AAACCCGCAG GT 42

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

NCUGANGANG UUGAAAN 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

NCUGANGANG UCGAAAN 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

NNNNNNNCUG AUGAGTTTTG AAACNNNNN 29

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

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

UCCUGUCUUG CAUUG 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGTTTT GAAACAGGA 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGTTTT GAAACAGGA 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GACGACCTTG AAACAGGA 28

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGANGAAA CAGGA 25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

GGAAGUCAGC CUA 13

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

GTCCTAGGCT CUGAUGAGTT TTGAAACTTC CTGGA 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

GTCCTAGGCT CUGAUGATTT TGAAACTTCC TGGA 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

UUCCAUGUCG GCAGAAT 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

ATTCTGCCCU GAUGAGTTTT GAAACATGGA A 31

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

ATTCTGCCCU GAUGATTTTG AAACATGGAA 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

ATTCTGCCCU GAUGAGGTTT GAAACATGGA A 31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CCAGGCAGUC AGAUCAUCUT 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

AAGATGATCT CUGAUGAGTT TTGAAACTGC CTGG 34

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGATGATCT CUGAUGATTT TGAAACTGCC TGG 33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

NNNNNNNNNN NNNNNNN 17

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

ATTCTGCCGA CATGGAA 17

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

ATTCTGCCCU GAUGAGTTTT GAAACATGGA A 31

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

ATTCTGCCCU GAUGAGTTTT GAGACATGGA A 31

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

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

UCCUGUCUUG CAUUG 15

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGUCCU UUUGGACGAA ACAGGA 36

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGTTTT GAAACAGGA 29

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGTTTT GAGACAGGA 29

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CAATGCAAGA CAGGA 15

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

ACGAAAGACG CTAAG 15

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GACGACCTTG AAACAGGA 28

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

UCCUGUCUUG CAUUG 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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

CAATGCAACU GAUGAGTTTC GAAACAGGA 29

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CAATGCAACU GAUGAGTTTT CGAAACAGGA 30

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CAATGCAACU GAUGAGUCCU UUUGGACGAA ACAGGA 36

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CAATGCAACU GAUGAGTCCT TTTGGACGAA ACAGGA 36

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CAAUGCAACU GAUGAGUUUU CGAAACAGGA 30

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAAUGCAACU GAUGAGUCCU UUUGGACGAA ACAGGA 36

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGAAGUCAGC CUA 13

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

UCCAGGAAGU CAGCCUAGGA 20

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GTCCTAGGCT CUGAUGAGTT TCGAAACTTC CTGGA 35

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTCCTAGGCT CUGAUGAGTT TTCGAAACTT CCTGGA 36

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCCTAGGCT CUGAUGAGTC CTTTTGGACG AAACTTCCTG GA 42

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GCGAGUCCAC ACT 13

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AUUUGCGAGU CCACACUGGA G 21

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CTCCAGTGTG CUGAUGAGTT TTCGAAACTC GCAAAT 36

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CTCCAGTGTG CUGAUGAGTC CTTTTGGACG AAACTCGCAA AT 42

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CAAUGCAACU GAUGAGUUUU CGAAACAGGa 30

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CAAUGCAACU GAUGAGUCCU UUUGGACGAA ACAGGa 35

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

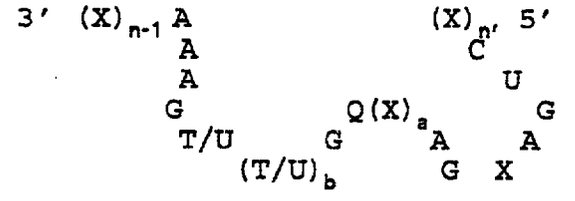
(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

UCCUGUCUUG CAUUG 15

WHAT IS CLAIMED IS:

1. A compound having the formula (Seq ID No. 3):



wherein each X and Q represents a nucleotide which may be the same or different and may be substituted or modified in its sugar, base or phosphate and Q represents guanosine;

wherein each of $(X)_n$ and $(X)_{n'}$ represents an oligonucleotide having a predetermined sequence which is capable of hybridizing with an RNA target sequence to be cleaved;

wherein each of n and n′ represents an integer which defines the number of nucleotides in the oligonucleotide;

wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof;

wherein a represents an integer which defines a number of nucleotides with the proviso that a may be 0 or 1 and if 0, the A located 5′ of $(X)_a$ is bonded to the G located 3′ of $(X)_a$; and

wherein $(T/U)_b$ represents an oligonucleotide with the proviso that b represents an integer which is 3 or 4.

2. The compound of claim 1, wherein the oligonucleotide 3'-$(X)_{n-1}$-A is 3'-$(X)_{n-2}$-C-A-.

3. The compound of claim 1, wherein $(X)_a$ is absent.

4. The compound of claim 1, wherein the integer b of $(T/U)_b$ is equal to 3.

5. The compound of claim 1, wherein the integer b of $(T/U)_b$ is equal to 4.

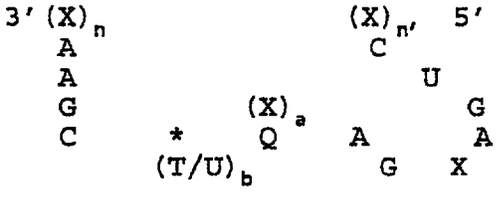
6. The compound of claim 1, wherein each X and Q is a deoxyribonucleotide.

7. The compound of claim 1, wherein each X is a ribonucleotide.

8. The compound of claim 1, wherein $(T/U)_b$ is a $(T)_b$.

9. The compound of claim 1, wherein $(T/U)_b$ is $(U)_b$.

10. A compound having the formula (Seq ID No. 4):



wherein each X and Q represents a nucleotide which may be the same or different and may be substituted or modified in its sugar, base or phosphate and Q represents guanosine;

wherein each of $(X)_n$ and $(X)_{n'}$ represents an oligonucleotide having a predetermined sequence which

is capable of hybridizing with an RNA target sequence to be cleaved;

wherein each of n and n' represents an integer which defines the number of nucleotides in the oligonucleotide;

wherein * represents a base pair between the nucleotides located on either side thereof;

wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof;

wherein a represents an integer which defines a number of nucleotides with the proviso that a may be 0 or 1 and if 0, the A located 5' of $(X)_a$ is bonded to the G located 3' of $(X)_a$;

wherein $(T/U)_b$ represents an oligonucleotide with the proviso that b represents an integer which is 3 or 4.

11. The compound of claim 10, wherein the oligonucleotide 3'-$(X)_n$- is 3'-$(X)_{n-1}$-A-.

12. The compound of claim 10, wherein the oligonucleotide 3'-$(X)_n$- is 3'-$(X)_{n-2}$-C-A-.

13. The compound of claim 10, wherein $(X)_a$ is absent.

14. The compound of claim 10, wherein the integer b of $(T/U)_b$ is equal to 3.

15. The compound of claim 10, wherein the integer b of $(T/U)_b$ is equal to 4.

16. The compound of claim 10, wherein each X is a deoxyribonucleotide.

17. The compound of claim 10, wherein each X is a ribonucleotide and GUUU(U)C is RNA.

18. The compound of claim 10, wherein $(T/U)_b$ is a $(T)_b$.

19. A composition which comprises a compound of claim 1 in association with an acceptable carrier.

20. A composition which comprises a compound of claim 10 in association with an acceptable carrier.

21. A oligonucleotide transfer vector containing a nucleotide sequence which on transcription gives rise to the compound of claim 1 or claim 10.

22. The transfer vector of claim 21, wherein the transfer vector is a bacterial plasmid, a bacteriophage DNA, a cosmid, or an eukaryotic viral DNA.

23. The oligonucleotide transfer vector of claim 21, wherein the oligonucleotide transfer vector is a plant DNA virus, a geminivirus or an infective phage particle.

24. The oligonucleotide transfer vector of claim 21, wherein the oligonucleotide transfer vector is packaged and contains the promoter sequences for RNA polymerase II or RNA polymerase III.

25. A host cell transformed by the transfer vector of claim 21.

26. The host cell of claim 25, wherein the host cell is a prokaryotic host cell or an eukaryotic host cell.

27. The prokaryotic host cell of claim 26, wherein the prokaryotic host cell is an E. coli host cell.

28. The eukaryotic host cell of claim 26, wherein the eukaryotic host cell is a monkey COS host cell, a Chinese hamster ovary host cell, a mammalian host cell or a plant host cell.

29. A method of cleaving a target mRNA in a subject which comprises administering to the subject an effective amount of the compound of claim 1 or 10.

30. A method of claim 29, wherein the administration is topical.

31. A method of claim 30, wherein the topically administered amount is between 1 ng and 10 mg.

32. A method of claim 29, wherein the administration is systemic.

33. A method of claim 32, wherein the systemically administered amount is between 1 ng and 500 μg/kg weight/day.

34. A method of claim 29, wherein the administration is by aerosol.

35. A method of cleaving a target mRNA in a host cell which comprises administering to the host cell an effective amount of a compound of claim 1 or 10 or

a transfer vector which on transcription expresses the compound.

36. The compound of claim 1 or 10 which further comprises an antisense nucleic acid which is capable of hybridizing with an RNA target sequence.

37. The compound of claim 1 or 10 which further comprises at least one additional non-naturally occurring oligonucleotide compound which comprises nucleotides whose sequence defines a conserved catalytic region and nucleotides whose sequence is capable of hybridizing with a predetermined target sequence. The compound of claim 37 wherein the additional non-naturally occurring oligonucleotide compound is a hammerhead ribozyme, a miniribozyme, a minizyme, a hairpin ribozyme, a hepatitis delta ribozyme, an RNAase P ribozyme or a combination thereof.